

The dangers of the university business

Universities are not businesses; attempts to run them as if they were risk trouble, as with two minor embarrassments at new British universities.

CAN a university qualify as a business in any ordinary sense of the word? Formally, there is no difficulty. A university is a corporate entity that may earn fees from its customers (its students) and may also award them qualifications at the end of a period of study. If it manages its affairs efficiently, it may also keep its employees in a state of relative prosperity and even make a profit (which, in most places, will not be taxed because of rules exempting 'not-for-profit' institutions from taxation). So why not, as most public authorities now seem to wish, attempt to turn universities into strictly entrepreneurial institutions? It is a fair question, but also a nonsensical question. University systems vary across the world, but all of them (even the private Japanese universities) are openly uncommercial in the way in which they organize their affairs. In comparison with other kinds of businesses, they are ostentatiously unenterprising.

In most places, for example, the number of young people wishing to become physicians has for many years exceeded the number of vacant places at medical schools. Properly organized businesses would respond to this demand either by increasing the fees charged to medical students far above the cost of educating them or would expand their medical schools to cater for the unmet demand. Instead, they timorously erect needless obstacles in front of would-be customers, requiring them to pass intellectually demanding tests and, sometimes, even demanding that they should demonstrate the personal qualities required of a physician, turning away good money in the process. It is as if the universities had undeclared objectives (to do with the quality of medical practice or, even worse, with the need for a social diversity of physicians) that improperly intrude on the proper customer-contractor relationship between a student and his or her client university. Medical schools everywhere should watch out for the anti-trust and anti-competitiveness legislation designed to end distortions of the market for reasons extraneous to it.

Value

A truly enterprising university or department would go further. It would say that the value of a university education is not just that of the piece of paper on which the qualification is written, but the value added to a person's intellectual capacity (and thus earning power) by the educative process. So why not forgive a student's fees, perhaps after a trial year, in return for a share of his or her lifetime earnings? There would be cash-flow problems, of course; teaching would

have to be paid for on the nail, while the 10 per cent or so of earnings would accumulate only in the future. But if only a market could be developed in ex-students' financial obligations to universities (ESFOUs they might be called), banks would no doubt be willing to bundle them together and sell them to the people called investors just as if they were mortgage bonds. Why have not the businesslike universities followed that route?

Nonsense

Because, of course, it is a nonsense. It is especially so when universities are sponsored by governments (regional or national) by means of direct support (for salaries, running costs and buildings) or for students (through reduced tuition costs and even maintenance grants). Far from being anti-competitive, the objectives extraneous to the customer-contractor (student-university, in that order) relationship are a large part of the reason why they exist. Teaching students is their bread and butter, doing that well is something else, the pursuit of scholarship a further extra.

These homilies are relevant because the British is not the only government in a muddle over its policy on universities. Two years ago, it created 25 new universities at the stroke of a pen by designating institutions called polytechnics as universities. This journal approved, partly for the sake of the diversity of higher education offered and partly because the polytechnics were hampered in their development by their lack of status. Unfortunately, the change coincided with one of the peaks of the government's rhetoric about the virtues of institutions of all kinds conducting themselves as if they were businesses.

Errors

Now, two of the new universities have fallen out with their vice-chancellors (presidents). One university has been told that it cannot carry through its decision to pay its departing head £400,000, in another the person has resigned after complaints that he was dividing his time between one big full-time job (for the university) and another 100 miles away, and that there had been errors in his expenses claims. No doubt that sort of thing happens all the time in business (the 'golden handshake' is certainly *de rigueur*), but it makes a nonsense of the notion of a university. Having created 25 of them overnight, the British government may now have to think through what it expects of them.

A little imagination will show that these incidents may be



a warning of worse to come. British universities are not the only ones recently to have been exhorted to create wealth, and to be seen to be doing so. The result has been that academic enterprises have flourished. Some have been excellent. (The investment of Trinity College, Cambridge, using private funds, in a neighbouring science park is an illustration of success.) But there is every likelihood that some universities will have backed industrial enterprises of various kinds that will then fail. There is a general understanding (sometimes a flat prohibition) that public money should not be put at risk in ways like these, but borrowed money is also money, and can just as easily be lost. What happens when such an enterprise goes to the wall, putting a university in the wrong with a group of angry creditors? And what will the consequences be for the educational enterprise that should be its true business?

What the academic enterprise therefore needs is a proper understanding of its obligations to its financial sponsors and some mechanism for making sure that their intentions are reflected in the rules with which it is required to comply. Governments (national or regional) as sponsors are usually clear about their intentions; universities exist to educate young people, admittedly in fields that the sponsors rather than the universities judge to be socially and economically beneficial. (British universities are now paid a fee per student that varies from one field to another, and which is essentially decided by the government's civil service, not those who have been seconded to the Universities Funding Council.) The government is purchasing a service (higher education) from its universities, presumably because it has been convinced that the result will further national interest.

Research on this logic is something else again. That is the commodity whose supply universities wish to see increased. Governments are on the whole indifferent, but they have been brainwashed over decades by academics protesting that teaching will be the worse if not accompanied by research, and that research is in any case the seedcorn from which productive innovation springs. So governments go along with the proposition, saying that if they must spend money on research, they want the seedcorn bit not the 'blue skies' bit. But not having the time themselves, they appoint businessmen to tell the difference for them. That is a good description of how the British government has been behaving in the past 15 years. Mr Newt Gingrich will soon be saying much the same in the United States unless President Bill Clinton says "no" more often than seems likely.

The trouble with businessmen is not that they are unimaginative, but that the timescale of their imagination is so much shorter than that of people who have been drawn into academic life by their interest in how the world is made. To say that is not to acknowledge that academic researchers' projects will bear fruit only ten years or more ahead — there has hardly ever been a time when the delay between the conception of an idea and its profitable execution has been so short — but that businessmen choose between different routes to the same goal by making crude estimates of the journey time. They rarely calculate the alternative values of the prizes that will await them. In business language they pay

less attention to the balance sheet than to the profit-and-loss account and sometimes even less to that than to the cash flow. Their inclination to take expensive short cuts when it seems to them that their 'time is money', probably explains why two new British universities have been deeply embarrassed. The concept of a university as a business has also taken a tumble (which does not give universities a licence to be unbusinesslike in their own affairs). Let us hope that when governments abandon their model of the universities as a business, not too many academics will be disadvantaged. □

Apaches against stars

There is no merit in the campaign of the Apache Survival Coalition against the Mount Graham telescope.

A letter from the self-styled "Apache Survival Coalition" appears on page 589 of this issue. This group, which admits in court documents that it does not represent the views of the Apache nation, a tribe of native Americans in the southwestern United States, has recently gone further. It has been sending hate mail to all and sundry in an attempt to block the development of an observatory near the top of Mount Graham, in southern Arizona.

Three native shrines, dating from the twelfth century, were identified on Mount Graham during the course of an environmental impact survey by the would-be builders of the telescope. All southwestern tribes, including the San Carlos Apaches, were informed in 1985 of the discoveries. Four tribes responded, but the San Carlos Apaches were not among them. The Zunis and Hopis expressed interest in the shrines, as they appear to be attributable to ancestors of those tribes, but felt that the proposed observatory would not affect the shrines (the closest of which is about a half mile from the observatory). The tribal council of the San Carlos Apaches took an officially neutral position on the observatory in July 1993. (In the tribal records, the decision is listed as JY-93-127.) That neutrality is appropriate; the San Carlos Apaches first migrated to southern Arizona during the 1600s.

The more lurid claims of the Apache Survival Coalition, which began its activities in 1989 when it became clear that the presence of the indigenous Mount Graham red squirrel would not prove an insuperable obstacle to the development of the observatory, include charges of "cultural genocide" against agencies of the German government. A recently distributed flyer claims that the building of the telescope is comparable with the Holocaust. These charges, combined with a campaign of misinformation, stand in stark contrast to the fact that the Apache Survival Coalition has not objected to the roads, summer homes and microwave transmitters on the mountain.

Can it be significant that names associated with the controversy over the effect of the observatory on the red squirrels (see *Nature* 372, 215-216; 1994) are also associated with the Apache Survival Coalition? On the face of things, the group seems to have a clear agenda — to block the observatory at all costs. It is a pity for the Apache Survival Coalition that it does not have a leg to stand on. Not even one. □

LHC is set for approval by CERN— if France agrees to play ball

London. With a critical meeting of the council of the European Laboratory for Particle Physics (CERN) due to take place tomorrow (16 December), laboratory officials are cautiously optimistic that they will at last receive the official go-ahead for the construction of CERN's Large Hadron Collider (LHC).

But no-one is counting any chickens before they are truly hatched. At the beginning of the week, it remained unclear whether the package would be acceptable to France.

In particular, the French had backed down from an apparent commitment a week previously to pay a sufficiently high 'host country premium' to satisfy both Germany and Britain.

Over the past few months, Christopher Llewellyn Smith, CERN's director-general, has drawn up a complex package of financial arrangements for the construction of the SFr2.6-billion (US\$1.6-billion) collider, which has already been approved in principle by both Germany and Britain — the laboratory's two largest contributors.

A key element is the premium that both France and Switzerland are being asked to pay, in recognition of the extra benefits they will obtain from the LHC, which will straddle the border between them. A visit by Llewellyn Smith to Paris last week apparently failed to resolve the issue.

But CERN officials were still hoping as *Nature* went to press that a compromise would be reached by the CERN council's executive committee when it meets in Geneva on 15 December. And that this committee would then recommend the construction of the LHC to the full council, which meets the following day.

If France still balks at the German/British demand, the LHC will be in serious trouble. Both CERN officials and physicists in member states warn that the whole project

ain) and France over the size of the host country premium.

At the time, Germany was insisting that Switzerland and France should jointly contribute 10 per cent of the anticipated cost of the construction of the LHC — SFr260 million — in recognition of their host nation status, in line with accepted practice with other large-scale European facilities.

Switzerland was reported to be prepared to go along with this. But French research officials, whose budget was already under

heavy pressure, said they could go up to only 5 per cent, or SFr130 million (see *Nature* 369, 510; 1994).

Last month, with its federal elections out of the way, Germany indicated that it was prepared to be more flexible. In particular, it reduced its demand for the extra premium to SFr170 million, indicating that the final figure was open to negotiation.

At the same time, however, Germany put forward various other conditions for its support of the LHC — each of which implied considerable new commitments by both CERN and its member states.

First, German officials said that, given their domestic financial difficulties they wanted the CERN budget kept level in constant money terms up to 1998, and thereafter to grow at a rate of only one per cent a year.

Second, they proposed that CERN itself should increase the resources being committed to the LHC by finding an extra SFr600 million from cutting back other activities. Finally, it wanted a clear commitment to SFr500 million from non-member states (in particular Japan and the United States) as the level of contributions that they would be prepared to make to participate in the construction and operation of the new machine.

The harshness of the German proposals led to some strong protests by CERN staff in Geneva. At the same time, however, they appear to have given CERN officials a clear indication of the scope for negotiation — and an outline of the package that might eventually be acceptable to all sides.

It is this package that Llewellyn Smith has been taking to European capitals in recent weeks, and which — following its apparent endorsement by research ministers of Britain, Germany and France at an informal meeting two weeks ago — was due to be presented to the committee of council today (15 December).

The new package is thought to include SFr160 million as the host country premium, with an 'indexation' of one per cent ►



Success story: A string of LHC superconducting magnets, which last week proved that they can run at a field of 8.36 tesla for 24 hours

could rapidly lose both scientific and political momentum (in particular through dwindling interest from the United States and Japan) that could take several years to build up again.

CERN is being more cautious about the outcome than it was in June. Then, its openly expressed confidence that LHC would receive the blessing of member states was scuttled by an unexpected last minute disagreement between Germany (backed by Brit-

Bid to raise interest in research

London. Two of Britain's research councils this week launched a £1-million, three-year initiative designed to develop a "lasting interest and enthusiasm" in engineering and scientific research among pupils between the ages of 14 and 16.

The so-called 'Pupil Researcher Initiative' is being launched jointly by the Particle Physics and Astronomy Research Council (PPARC) and the Engineering and Physical Sciences Research Council (EPSRC). Coordinated by the centre for science education at the Sheffield Hallam University, its central plank will be 30 pupil research briefs (PRBs).

These will be small-scale investigative projects based on topical research being funded by the two councils. PPARC and EPSRC researchers will work with school teachers to link the projects with relevant areas in the recently reviewed national curriculum (see *Nature* 372, 211; 1994).

Once the research briefs have been compiled — probably in early 1996 — materials will be sent on computer disk and in hard copy to every secondary school in the United Kingdom. The research councils hope that science teachers will chose three or four briefs to use with their pupils, "encouraging active learning and teamwork".

The PRBs will be supported by various elements intended to give pupils first-hand experience of "what it is like to be a researcher", including the possibility of having their research published in a special *Pupil Research Journal*, and presenting results at regional conferences.

The initiative will also sponsor teacher placements in research laboratories, and provide small bursaries for up to 1,000 PPARC and EPSRC postgraduate students to link up with a local school in order to give talks, work with teachers and help with projects. □

► less than the rate of inflation after 1998, when a full assessment would also be made of commitments from non-member states, and thus of the likely completion date.

Furthermore, there appears to have been agreement already on a new voting procedure in the CERN council that would give Germany greater power to veto future spending plans.

A new procedure would require all spending to be approved by countries contributing at least two-thirds of the budget; in practice, this means it could be blocked by Germany and one other major contributor (such as Britain), as such a combination provides more than one-third of the total budget.

German and British officials are confident that the chances of agreement are high. But the outcome remains dependent on France. One compromise being suggested on Monday was that, rather than accept the full demand for the host country premium, France could agree to make up the one per cent gap between the proposed budget increases after 1998, and the real inflation rate.

If France rejects any such compromise, its action would cast significant doubts on its credentials as a supporter of high-level European science. Conversely, although the German pressure appears to have provoked some resentment (not only in Paris), observers say that Germany is sufficiently confident in its approach to be prepared to weather any storms it may have provoked.

Indeed, given that Germany is also the biggest contributor to CERN's budget, even after a recent temporary reduction because of the costs of reunification, there is a pragmatic acceptance that, as one official puts it, "he who pays the piper calls the tune".

David Dickson

Figures reveal Europe still falling behind US research

Paris. The European Commission has produced for the first time a single volume of science and technology indicators dedicated to describing research in the European Union (EU) and its place in the post-Cold-War world. In particular, the report — a brainchild of Antonio Ruberti, the outgoing research commissioner — shows that the United States has "recovered" its technological lead over the past three years, while new Asian economies are now "catching-up".

At the same time, it reveals that Europe's reservoir of scientists and engineers is only about half that of the North American Free Trade Association. The report warns that this may decline further in comparison to North America and South-East Asia — and that, without a sustained economic recovery, Europe will increasingly have to select its priorities and slim its research organizations.

The 337-page volume was prepared in less than six months by the commission, in association with five science policy units (one in Maastricht, the Netherlands, one in Paris, and three in the United Kingdom). Luke Georgiou of the Programme of Policy Research in Engineering Science and Technology (PREST) at the University of Manchester describes it as the "most comprehensive" analysis of European science so far. This first report will be updated regularly within the EU's

new socio-economic research programme.

In addition to flagging the overall distance that Europe trails behind both Japan and the United States, the report paints a bleak picture of European competitiveness in computing and electronics, illustrating how, despite EU programmes such as ESPRIT, the gap with both the United States and Japan is widening. Europe, however, remains strong in pharmaceuticals, aerospace and automobiles, and is holding its own in telecommunications.

The report confirms that the high growth of research budgets during the 1980s in Europe has been replaced in the 1990s with cutbacks because of the recession, and points out that these falls have been most marked in the public sector.

Another trend is a general reduction on research spending on energy and defence. At the same time, there has been an increase in research orientated towards socioeconomic objectives such as health and the environment.

The report also emphasizes the growing trend of globalization of research, with an increase in internationally co-authored papers, and in the number of overseas company laboratories.

Declan Butler

● The European Report on Science and Technology Indicators 1994 (EUR 15897 EN) (ISSN 1018-5593).

Nuclear industry seek US/Europe agreement on export controls

London. The international nuclear fuel industry is putting pressure on the United States to break a deadlock in negotiations over a new Nuclear Cooperation Agreement with Euratom, the European nuclear agency, to succeed the current agreement, which expires at the end of 1995.

The main sticking point is the United States' insistence on retaining 'prior consent rights' on nuclear material of US origin, in line with the Nuclear Non-Proliferation Act (NNPA) signed in 1978 by President Jimmy Carter.

As the US-Euratom Nuclear Cooperation Agreement, signed in 1958, pre-dates the NNPA, conflict with Europe has so far been avoided by an annual waiver issued by the US president. But Congress is unlikely to agree to a long-term waiver.

As a result, the United States has therefore offered Euratom terms similar to those in its bilateral agreement with Japan. This includes 'programmable approval', which effectively means that use of nuclear material, equipment or technology of US origin has to be agreed by both parties before

transfer occurs.

But this proposal has not been welcomed by the 12 member states of the EU. "The idea that the United States has a supervisory role is unacceptable to them," says Clark. In March this year the EU's Energy Commissioner, Abel Matutes, wrote to Warren Christopher, the US Secretary of State, saying that the United States' prior consent requirements were unacceptable.

In explaining this decision, Matutes pointed out that "Western Europe and the USA are two equal partners who have attained a comparable leading position in the use of nuclear energy, and are equally strongly committed to non-proliferation."

The Uranium Institute (UI), which represents 80 companies in 18 countries — including uranium mining companies, electrical utilities, fuel processing and trading companies — says that failure to reach agreement would be a major setback for the whole nuclear industry.

The immediate consequences will be the interruption of nuclear exports from US companies to the European Union (EU). But

Gerald Clark, secretary general of the institute, says the knock-on effects could be even greater.

In particular, third countries would be prevented from transferring to the EU any nuclear material that is US-obligated, that is, any fuel that has been mined, enriched, fabricated or converted at some stage in its life in the United States. In addition, Clark warned last week, continued investment in nuclear power could be jeopardized by a lack of security in long-term contracts.

Failure to reach a new agreement could also threaten negotiations over extending the Nuclear Non-Proliferation Treaty (NPT), due to take place next year. The UI fears that such a failure might be interpreted by the rest of the world as a "complete breakdown" in nuclear cooperation between two major nuclear trading areas.

The nuclear industry, according to the Uranium Institute, would be happy to see an extension of the current system. "The fact is that the existing arrangements, although untidy, do work," says Clark.

Maggie Verrall

EPA reveals plans to double external grants

Washington. Keen to improve the quality of the science it sponsors in universities and elsewhere, the US Environmental Protection Agency (EPA) plans to double spending on grants to outside researchers next year, to \$44 million.

Most of the additional \$22 million will pay for environmental research funded jointly with the National Science Foundation (NSF) in four areas of mutual interest: water and wetlands, global change, pollution prevention and social dimensions of environmental issues. The rest of the money will be used for grants in areas of particular interest to EPA, such as indoor air pollution.

Under an agreement signed by the two agencies last week, NSF will take the lead in peer-reviewing both types of grants. The call for proposals is due to go out next month.

EPA hopes the expanded grant programme will strengthen both its ties to academic scientists and its research base. Robert Huggett, the new head of the agency's Office of Research and Development (ORD), says the agency would like to double its funding for extramural grants again in 1996, and to reach \$100 million by 1997.

Huggett also hopes that the link with NSF will give greater credibility to EPA's peer-review process, long criticized for its lack of rigour. Indeed, Congress has told NSF to help the agency with its peer-review procedures, and made the release of next year's funding for ORD contingent on a further review of agency research by the National Academy of Sciences.

Huggett says he is familiar with the poor reputation of EPA research from a period he spent on the agency's science advisory board. Now, he says, he is working to remove that stigma and make EPA "the first choice for a young [environmental] scientist or engineer right out of school".

To enlarge the pool of available talent, the agency is also initiating a new graduate fellowship programme. This will fund 100 master's and PhD students in 1995, with a target of 300 students by 1997. The call for applications for next year's fellowships went out this week.

In his first few months in the job, Huggett has earned high marks for his energy and enthusiasm for reforming EPA science. But he faces formidable institutional obstacles, not least a fundamental conflict between the agency's regulatory mission and its desire to carry out long-term research.

In the past, EPA scientists have been diverted from research projects to provide quick answers whenever regulators need to write a new rule on, for example, clean water or pesticide safety. Huggett says this primary mission will never go away. "ORD exists to support the programme offices," he says. "It does not exist to conduct environmental research for the sake of it."

But he says he also wants to create a more hospitable climate for scientists working on long-term research problems. ORD will now have a dual promotion track, so that bench researchers can advance as quickly as scientists who manage contracts and handle administrative duties.

Some agency scientists who have been doing administrative work will be offered schooling to "get back into the mainstream of science and engineering". And Huggett wants to begin a competitive internal grants programme, also peer-reviewed by NSF, that would allow projects proposed by EPA's own scientists.

Agency officials have made it a goal to increase the amount of long-term research at EPA from the current one-third to a half of the agency's total research budget to half (see *Nature* 370, 239; 1994).

Perhaps the most visible change within ORD is the restructuring of the office's 12 research laboratories into four 'mega-labs':

two in North Carolina focused on environmental health effects and exposure to those effects; a national risk assessment centre in Washington; and a centre for risk management in Cincinnati, Ohio. The reorganization should be completed by next month.

So far, the reform of EPA science is largely limited to shuffling existing resources. The head of the agency, Carol Browner, has deferred any decisions about closing or consolidating laboratories until 1996. And although more than 250 contractors will become employees, they are mostly laboratory technicians, not scientists.

Huggett has so far been able to find the money he needs for next year's expanded grants programme by 'reprogramming' research funds that had been used to support the regulatory offices. But reaching his target of \$100 million will almost certainly require additional funds — which the new Congress may be reluctant to provide.

Tony Reichhardt

DNA, sex and the single macaw

London. Scientists at the University of Oxford are using the polymerase chain reaction (PCR) in the fight to save the world's most endangered bird, the Spix's macaw *Cyanopsitta spixii* (right), by developing a test to establish the sex of the only Spix's macaw still in the wild.

The species was believed to be extinct in the wild until a single bird was spotted in the arid interior of east-central Brazil in July 1990. The Brazilian government established a Permanent Committee for the Recovery of Spix's Macaw in the mid-1980s. This consortium, which includes most holders of captive birds, now plans to release one of the 26 known captive birds as a mate.

The problem is that no-one knows the sex of the lone wild macaw. According to Martin Kelsey, head of the Americas division at BirdLife International, evidence from field studies suggests that the wild bird is a male. The consortium has therefore decided to release one of the very precious females originally captured from the wild as its chances of survival are higher. The bird is likely to be released in December, when food is most abundant and pair formation is most likely to take place.

Around two-thirds of the world's bird species cannot be sexed visually, so the usual technique is a chromosome squash, using material from the base of a growing feather. This would involve capturing the wild bird, a traumatic experience that the Brazilian government wanted to avoid.

As a result, Richard Griffiths and Bela Tiwari from the Unit of Ecology and Behav-

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iour have turned to PCR to amplify the small amounts of nuclear DNA contained in moulted feathers collected from the jungle. But they also need a marker.

In birds, the female is heterogametic, having one W and one Z chromosome, while the male has two Z chromosomes. Using DNA from chickens, Griffiths and Tiwari identified a W-linked sequence which, according to Griffiths, is probably common to all birds.

They focused their search for the marker using a genomic library from the related species, the hyacinth macaw *Anodorhynchus hyacinthinus*, and isolated a W-linked portion of the parrot genome. The test has been 100 per cent accurate when tested on captive Spix's macaws.

The researchers have also confirmed that the marker is W-linked using Southern blots. "We've got the test. The difficulty is getting enough DNA out of the feathers," says Griffiths. Maggie Verrall

Coalition pursues ban on animals in teaching

Munich. The local government of Hessen, one of Germany's 16 *Länder*, has confirmed its commitment to eliminating the use of animals from undergraduate teaching at its universities. Two weeks ago, it told a professor of zoology at the University of Marburg, Gerald Heldmaier, to stop using animals in his courses.

The move has come despite a ruling by an administrative court last December that the country's animal protection laws could not be used to restrict the freedom of university teachers, which is protected by Germany's federal constitution, from outside interference (see *Nature* 367, 103; 1994).

Last year, Heldmaier, with the support of the University of Marburg, persuaded a local administrative tribunal to issue an injunction against an earlier attempt by the Hessen government to impose a general ban on the use of animals in teaching. This was subsequently upheld by the state administrative court.

The government, made up of a 'red-green' coalition of Social Democrats and Greens, says that it has further evidence to support its demand that Heldmaier should stop using animals in his course.

But the government itself appears to be split on the issue, as the state's ministry of science has at the same time told the University of Marburg that it can pay the costs of any legal action to defend Heldmaier out of its state funds.

Heldmaier, who intends to appeal against the government's new ruling, has vociferously defended his right to include a single animal experiment in his zoology under-

graduate course. This involves a study of the intestinal absorption of glucose from the intestines of anaesthetized rats.

He argues that zoology students should have practical experience of the control of anaesthesia, as well as practical knowledge of simple surgical techniques. But opponents claim that the same information can be derived from showing students videotapes of the operation (see *Nature* 365, 778; 1993).

Heldmaier claims that, if the Hessen

government wins, it could set a precedent for the state government to interfere in other areas of university teaching. But the conflict has arisen from the fact that the use of animals in teaching is strictly limited by the German Animal Protection Act.

"This is a political principle, and a very tense question," says a spokesman for the state government. "If necessary we will defend our decision right up to the highest court, the federal constitutional court [in Karlsruhe]."

Alison Abbott

Livermore head promises openness

San Francisco. **C. Bruce Tarter, appointed last week as the new director of the Lawrence Livermore National Laboratory in California, says that he plans to lead the science institution into an era of more openness and greater interaction with both the US public and the technical community.**

Tarter, who is 54, has been acting director since last May, when he succeeded John Nuckolls. An astrophysicist, Tarter has worked at the laboratory for 27 years. "I believe the Livermore Laboratory is the outstanding applied science institu-

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Tarter: faces large challenges ahead.

Lawrence Livermore Laboratory

tion in the country, and it will be my job to ensure that its capabilities are applied to the nation's highest technical priorities," he told a news conference at the laboratory last week.

Tarter takes over the directorship of Lawrence Livermore at a time when the national laboratories face potential dismantling of their sponsor, the Department of Energy, as well as threats of severe budget cuts from the new Republican-dominated Congress. A report commissioned last year by Hazel O'Leary, the Energy Secretary, on the management of US weapons laboratories is due to be completed in February.

As a result of such pressures, Tarter is likely to be called upon to cut spending — perhaps by redeploying staff — and to justify the laboratory's varied programmes. As acting director, Tarter has already encouraged closer collaboration with industry, and has declared a goal of making the laboratory more "user-friendly".

Tarter said last week that the ending of the Cold War meant that it was time for the laboratory to revise its mission. He emphasized its potential for carrying out important research and development work in energy, the environment, bioscience and health care.

One scientist familiar with the laboratory says that Tarter has already demonstrated as acting director an ability to convey the importance of the laboratories to the public, and that his commitment to openness and communication with employees is also paying off, for example by improving the dedication and productivity of staff. Both public and staff support are likely to be critical in the laboratory learns to adapt to a changing environment.

Tarter was appointed by the University of California Board of Regents, which manages the laboratory for the Energy Department. He is the tenth director since the laboratory was founded as part of the US nuclear weapons programme in 1952.

Sally Lehmann

Moscow meeting raises research fund hopes

Washington. A joint US-Russian research foundation first proposed more than two years ago (see *Nature* 355, 576; 1992), may finally get off the ground at a meeting this week in Moscow between the US Vice President Al Gore and the Russian Prime Minister Viktor Chernomyrdin, according to White House officials.

The meeting will also attempt to solve problems with projects that the two countries have already set in motion. These include difficulties in getting scientific equipment through Russian bureaucracy and customs and in allowing scientists access to sensitive areas.

More than two years ago, at the instigation of Representative George Brown (Democrat, California), Congress approved the creation of a joint US-Russian research foundation, to be administered by the National Science Foundation. So far, however, the money has failed to materialize.

The Department of Defense (DoD), which was to have paid some \$10 million into the so-called 'Brown Fund', has been reluctant

to part with the money, and the White House has shown no willingness to force its hand. "I don't understand why the administration has not done this," complains one frustrated congressional staff member who worked to pass the legislation.

The DoD has previously claimed that the transfer of funds would be illegal. But Jeff Schweitzer of the White House Office of Science and Technology Policy says he hopes the legal questions can be settled in time for the meeting on 15 and 16 December.

The meeting between Gore and Chernomyrdin, part of a continuing series of talks on cooperation between the two countries, is also expected to initiate several new joint projects. These include a space biomedical centre in Moscow funded largely by the US National Aeronautics and Space Administration, biodiversity studies, research on nuclear waste processing, and a project to measure ocean temperature in cooperation with the Advanced Research Projects Agency.

Tony Reichhardt

Australia's CSIRO takes the flak from Senate committee

Sydney. The management and board of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia's leading research organization and its largest employer of scientists, have come in for some harsh criticism from a parliamentary committee.

A report released last week by the Economics Reference Committee of the Australian Senate describes the board of CSIRO as "ineffectual". On the basis of hearings held in Canberra, the federal capital, over the past few months, the committee claims that the board allowed the organization to become over-managed, with an "archaic, hierarchical, top-heavy management system", to the point where research became badly affected.

The committee's report also says that the board had neglected the employment conditions and morale of its staff and taken an "intrusive" role in matters of administration that should have been left to the organization's chief executive officer (CEO), John Stocker, and his executive committee.

At the same time, Stocker, who is a member of the board, is personally criticized for making a "tokenist response" to management problems raised by the Senate committee by simply pointing to the organization's adoption of the management discipline of Total Quality Management (TQM). Earlier this year, Stocker announced that he would not be seeking reappointment for another five year term as CEO, when his present term expires in March 1995.

The committee's inquiry was initiated by complaints from Australia's strong — but drought-affected — rural sector about a decision by the research agency to cut back funds for rural research. Announced earlier this year, the cuts are partly the result of changes in the organization's priorities as well as efforts to reduce the CSIRO's A\$126-million budget by 13 per cent (see *Nature* 369, 347; 1994).

The CSIRO is not obliged to act on any of the committee's recommendations. But the report is likely to have a strong influence on the government's deliberations over the state-owned organization's future.

As well as declaring that the cuts in funding for rural research should be reversed, the committee recommends that CSIRO should improve its efforts to commercialize research, reassess the effect of external funding policies on fundamental research and improve communications with the public.

But the report's main criticisms concerned the management of CSIRO in general and its institute structure in particular, which the committee recommended should

be abolished or modified.

CSIRO's main operating units are known as divisions which, in 1986, were grouped into six institutes of broadly similar research areas, such as the Institute of Animal Production and Processing, based in Sydney.

The committee had received a large number of submissions from individual scientists, which all strongly criticized the institutes as having no particular function. Rather than adding value to the research or commercialization process, the report said, institutes had become involved in matters of accountability and review, generating "a vast amount of paperwork without clear and worthwhile objectives".

The CSIRO has so far said little in response to this report. But an internal commit-



tee is now examining the organization's structure, and will report in February.

But the Senate committee's report also criticizes the management review committee, chaired by the chairman of the CSIRO, Adrienne Clarke. It found the terms of reference of the review to be "very general" and "so unfocused as to be almost incomprehensible". It points out that there had already been a series of management reviews that had led, not to any change in structure, but to a proliferation of corporate managers and administrators.

Among other points made by the report, the Senate committee notes that scientists had become frustrated by endless reviews of CSIRO (one scientist complained his area of research was reviewed four times in 12 months); that administrative staff appear to cost the same per person as research staff despite not having research programme costs; and that both science and management appeared to have suffered through the practice of promoting scientists to management positions.

Mark Lawson

Biodiversity treaty nations to study biosafety protocol

Nassau, Bahamas. The signatory states to the United Nations (UN) Convention on Biological Diversity formally agreed last week to study the need for a global biosafety protocol in the coming year.

At the conclusion of the first meeting of the 'conference of parties', held in Nassau in the Bahamas over the previous two weeks, they also agreed to examine the relationship between questions of access to genetic resources, technology transfer to developing nations and intellectual property rights.

A study commissioned during the preceding year of preparatory meetings had pointed out the need for a biosafety protocol to regulate the production and release of genetically modified organisms. Many delegates and representatives of non-government organizations (NGOs) were therefore disappointed by the insistence of several developed countries on reopening this question in the coming year. They would have preferred future meetings to consider the actual wording of such a protocol.

The United Kingdom and the Netherlands had proposed a set of interim guidelines on biosafety that have been formally adopted as a basis for future guidelines by the United Nations Environment Programme. But the proposal was criticized on the grounds that, since many developing countries have inadequate or non-existent biosafety laws, such guidelines would be voluntary — and hence easily ignored.

During the pre-conference negotiations, developing countries pushed for action on the issue of equitable access to, and development of, genetic resources of potential value to major industries such as pharmaceutical or agrochemicals — an issue raised explicitly in the Biodiversity Convention.

They argued that access to these resources should imply a quid pro quo that might include exchange of technology or other immediate benefits, as well as the sharing of royalties or inventorship. This could bring new economic incentives for the conservation and sustainable use of biological diversity.

One item that has therefore been adopted as part of the 1995 work programme for the parties to the convention is a study of intellectual property rights.

The 1995 programme also includes the need to consider national strategies to promote biodiversity conservation, which may eventually require all parties to formulate programmes of action. Parties to the convention will also consider the conservation of coastal and marine biodiversity.

But although forests are home to an estimated 60 per cent of the world's species, sustainable forestry is not included.

Daniel Putterman

Row over industry link-up splits staff of Spanish lab

Madrid. Three scientific directors and a deputy director have resigned their posts at the National Biotechnology Centre (CNB) in Madrid over the terms of an agreement under which laboratory space at the centre will be rented to Pharmacia Iberia, the Spanish subsidiary of the Swedish pharmaceutical company Pharmacia.

The centre belongs to Spain's Consejo Superior de Investigaciones Científicas (CSIC), and the agreement has opened a fierce debate on collaboration between the private sector and public research centres in Spain.

According to the seven-year agreement — which has yet to be ratified by Pharmacia's headquarters — Pharmacia Iberia will equip laboratories on one floor of CNB's four-floor building. It will also set up a joint research department in immunology and oncology with a research staff from both Pharmacia and CNB.

The overall deal is worth Pta 8 billion (US\$60 million). Equipping the laboratories will cost Pta 450 million, and the rest of the money will pay for the salaries of Pharmacia Iberia's research staff, and about 25 per cent of the general running costs of CNB.

In exchange, all patent rights on research carried out in the Pharmacia-CNB laboratory will belong to the company. According to the agreement CNB will be offered compensation "to be negotiated in good faith".

CSIC, whose budget has been frozen for five years (funding for the new centre ran out before its final floor could be fitted), has encouraged collaboration between public research centres and private industry both as a way of increasing research funding and of developing more market-orientated science and technology projects.

Many collaborative projects have been agreed. But that with Pharmacia Iberia is the first under which CSIC laboratories have been rented out and at the same time all intellectual property rights have been given to a private company.

José Mato, the president of CSIC, sees the Pharmacia Iberia agreement as a model for the future. "It is an innovative contract", he says. "We are taking some risks, but there are increased benefits, such as increasing interchange between basic research and technological development."

Such views are strongly endorsed by the new secretary of state for universities and research, Emilio Octavio de Toledo. He describes the agreement as a "well-balanced collaboration, the most important yet be-

tween CSIC and industry."

But many CSIC scientists are angry at what they feel to be industry's exploitation of a public institute, claiming that CSIC has given away too much to Pharmacia Iberia. Angel Pestaña, for example, who represents CSIC researchers on its governing board, says the agreement "does not conform to the normal formula to regulate collaboration among scientific institutions".

CSIC staff are also upset that neither the



in dispute: CSIC's National Biotechnology Centre.

head of the CNB nor its four scientific directors were consulted before the deal was made public at the beginning of October.

José Luis Carrascosa, one of the four scientists who resigned their administrative posts over the affair (although continuing to work as researchers at the institute), says that CSIC has reneged on a written promise to consult CNB before anything was signed.

Scientists are worried that Pharmacia Iberia may use the agreement as an excuse to close down its own industrial research and development centre.

There is a precedent. Pharmacia Iberia was formed in May 1993, when KABI-Pharmacia merged with the company Farmitalia Carlo Erba. A month before the merger, KABI and CSIC signed an agreement to set up an immunology unit at CNB, made up of both KABI and CSIC staff.

The agreement itself was not controversial. But shortly afterwards KABI closed its own research centre Instituto Llorente and dismissed half of its staff. Carlos Laró, a spokesman for Pharmacia Iberia, concedes that this agreement "re-orientated a line of research in immunology that we then discontinued at Llorente".

Laró adds that the company has not made an official decision on the future of its own R&D centre Antibióticos Farma. "We will have to coordinate their work with our work at CNB," he says. In his view, the deal is a positive one for CSIC, as "otherwise the floor at CNB would have remained empty".

Luis-Angel Fernandez Hermana

CNRS agrees to pay up over destruction of computer files

Paris. A French physicist has won a second legal round against the Centre National de la Recherche Scientifique (CNRS) in a seven-year fight to contest the way that the CNRS stripped him of his research funding and expelled him from its Nice observatory without any formal disciplinary procedure.

Gilbert Reinisch and his colleague Jean-Claude Fernandez were expelled in 1987 by Raymond Michard, then director of the observatory. Both immediately filed a lawsuit, and Philippe Delache, Michard's successor, reinstated the researchers, but working next to astronomers and not in their physics laboratory.

The conflict might have stopped there, but Michard also ordered the closure of the researchers' accounts at the super-computer centre at Montpellier without informing them. The files were subsequently destroyed; Reinisch claims they included software developed as part of a collaborative European Union research programme.

CNRS launched an internal investigation into the matter, which found in favour of the laboratory management. Moreover, Delache wrote to the director general of CNRS that "study of the report leads me to conclude that these researchers have no place in my establishment". In December 1990, he ordered both researchers to leave the observatory within two weeks.

Reinisch and Fernandez were subsequently sent to share an unequipped room in a geology institute 20 km away. Later in 1991, the administrative tribunal of Nice ruled that Michard's initial expulsion of the researchers had been an abuse of power and should be revoked. But CNRS has never reinstated the researchers.

In the latest twist, the same tribunal last month ruled that CNRS was responsible for the destruction of the computer files, and ordered the agency to pay each of the researchers FF15,000 (US\$2,800) for damage to their scientific reputation.

Reinisch condemns the fact that it has been left to him to prove improper treatment by CNRS, claiming that the years of legal procedures have irreparably damaged his career and reputation. The CNRS, for its part, admits that "there was an administrative mistake," but says that it is not in proportion to Reinisch's claim, and that the judgement "does not directly condemn the CNRS."

The final verdict will come next year, when the tribunal rules on the legality of the expulsion of the researchers, following the dispute over the computer files. Meanwhile, Reinisch has written to Guy Aubert, the newly appointed director general of CNRS, asking him to close the dispute and reinstate him and his colleague.

Declan Butler

UN AIDS chief will 'mobilize' commitment . . .

Paris. Peter Piot, the current director of research and intervention development of the World Health Organization's (WHO)'s Global Programme on AIDS, was this week appointed head of the United Nations Joint Programme on HIV/AIDS, which is scheduled to begin in 1996.

Piot was appointed by the heads of the six UN agencies that will take part in the new programme: the Children's Fund (UNICEF), Development Programme (UNDP), Educational Scientific and Cultural Organization (UNESCO), and Population Fund (UNFPA), and the World Bank and WHO. His appointment was confirmed on Monday by Boutros Boutros-Ghali, the UN secretary general.

Piot says his priority will be to "mobilize" political commitment to preventing AIDS. Such support has fallen over the past few years, he says, because of a combination of the economic recession and "AIDS fatigue", the latter caused partly by the absence of the predicted explosion of heterosexually transmitted AIDS in developing countries.

Maintaining the political momentum created by the recent AIDS summit will be the first task, according to Piot, who says this involves winning over not only heads of states but also the research community, non-governmental organizations and community leaders. The fact that there will be one channel for donor aid will make a big difference but the "acid test" of the new programme will be how it performs at the "country level".

Indeed, there is concern that the new programme may simply amount to cosmetic changes and that it will lack a coherent strategy. Piot argues, however, that there is no "magic bullet" against AIDS and describes the fact that the various UN agencies have very disparate ideas about the best approach to tackle AIDS as "no bad thing", providing that they are coordinated.

Piot says that the research priorities of

the new programme will include vaccine trials, vaginal microbicides and behaviour (see *Nature* 372, 308; 1994).

The choice of Piot has been welcomed by AIDS researchers. "He has a great track record, they couldn't have made a better choice," says one. But they are more sceptical of his chances of success, given the

political forces he will have to contend with.

In particular, although WHO does an excellent job in global epidemiological research, one AIDS researcher argues that the diplomatic imperatives that constrain WHO and other UN agencies render them "largely irrelevant" to the urgent and coherent action needed against AIDS.

Declan Butler

. . . as tighter research links urged

San Francisco. AIDS researchers need to work more closely with those providing services and developing policy, according to a task-force on the HIV epidemic set up by the city and county of San Francisco, where the disease first became apparent more than 10 years ago. "The kind of research that is going on does not necessarily help prevent infections," says Mitchell Katz, director of the AIDS Office of the San Francisco Department of Health and a member of the study group.

The joint task-force was made up of 25 people from a wide range of AIDS service organizations and city offices, and had been asked to evaluate HIV services and prevention in San Francisco.

In a report to Mayor Frank Jordan and the Board of Supervisors, the group has emphasized the importance of research in all aspects of the city's response to the epidemic. But it has urged researchers at the University of California, San Francisco, to work with community-based organizations and the Department of Public Health to set research priorities, disseminate information and raise research funding.

Most research in the United States occurs independently of the delivery of care, and there is very little interaction between the two, says Katz. "That is a major failing of our system. It results in the research being less pertinent to the policy-makers and the people who are offering the services."

Although there is a great deal of research on ways of predicting sero-conversion, Katz says there is relatively little study of the efficacy of attempts at prevention, even though this would be far more useful to the providers of HIV services.

The task-force has recommended more research into behaviour associated with HIV transmission, rather than emphasizing risk groups. Population-specific studies should give priority to women, ethnic minorities and young people, without reducing the focus on gay men, its report says.

Michael S. Ascher, acting chief of the California State Virus Laboratory in Berkeley, agrees with the task-force that interaction between researchers and service providers is critical for studies to be relevant to the community.

The task-force said that researchers need to work harder to address the changing demographics of AIDS as more women, homeless people and drug users become infected with HIV. In particular, it urges researchers to investigate woman-to-woman transmission of the virus, to develop technology to prevent oral-vaginal transmission, and to evaluate treatment protocols designed for men to see if they are appropriate for women.

In recent years, the National Institutes of Health have begun to require the inclusion of women and minorities in clinical trials. The National Institute of Allergies and Infectious Disease has particularly emphasized women and drug users in its vaccine preparedness programme to recruit a cohort for potential large-scale HIV vaccine trials.

Partly because those with AIDS are living longer than in the past, members of the task-force also asked researchers to look more closely at the merits of non-Western medical traditions. "The thrust of research is for scientific knowledge, not consumer-driven," said Daniel Toleran, chair of the Filipino Task Force on AIDS and a clinical social worker at the University of California, San Francisco.

The task-force concluded that lack of funding, the changing nature of the epidemic and the increasing need for long-term services are threatening the quality of San Francisco's system of AIDS care.

Sally Lehrman

MPG outlines new plans for eastern Germany

Munich. The Max-Planck Gesellschaft (MPG), Germany's leading basic research organization, plans to have up to 20 new research institutes fully operational in the new *Länder* by the end of the century, according to its president, Hans Zacher.

Addressing the MPG's annual meeting in Berlin earlier this month, Zacher said that the MPG aims to boost its activities in the east to the level of the west within a decade of reunification. Eight institutes have already been opened in the new *Länder*.

The organization will also support, for five years, 28 research groups — all but one within universities — to help boost the quality of their research, which had declined significantly during the commu-

nist years.

MPG will provide temporary support for seven new social sciences centres in the new *Länder*, planning to transfer these to universities within four years. It will also open eastern branches of the Garching-based institutes for extraterrestrial physics and plasma physics.

The money for these projects will come out of savings made by limiting the number of emeritus positions offered to three out of four scientists who become eligible for them, and limiting the number of research groups in the old *Länder*. The latter have a fixed life span and savings can therefore be realized relatively quickly.

Alison Abbott

Remarkable and novel increase

SIR — There has always been a striking difference between scientists and physicians given to flamboyant accounts of their own work and those who prefer a more modest account or telling understatement. With similar pieces of work, one group might go into print with a paper on “Intrinsic DNA polymerase activity of vegetable material extracted from *Hevea brasiliensis*”, while another group would startle us all with “New life out of old rubber tyres”.

Prompted by such considerations, I have recently scanned the biological literature (listed in *Index Medicus* and *Med-line*) for evidence of changing trends in the selection of evocative words used in the titles of papers. A 30-year period from 1966 to 1995 was considered in five-year blocks. The period 1991–95 has been calculated on the basis of the rates from January 1991 to August 1994, and then extrapolated to December 1995, assuming no change in the trends over that period.

The total number of publications between 1966 and 1970 was 986,670. This has increased in a more-or-less linear manner to a predicted total of 1,801,430 for the years 1991–95. During this period, the number of papers with the words *novel*, *new*, *exciting* or *remarkable* in the title increased also in a linear manner from 19,857 (1966–70) to 45,521 (1991–95). Other words, such as *reliable*, *secure*, *consistent* or *precise* might be considered equally evocative, although rather less strident. These words were used relatively infrequently, but they increased nevertheless from 204 (1966–70) to 1,004 (1991–95). It will be apparent that the use of all 8 words has increased more rapidly than the total number of papers over the same period. Furthermore, the increase in the use of type 2 words (*reliable*, *secure*, *consistent* or *precise*) has outstripped the increase in the use of type 1 words (*novel*, *new*, *exciting* or *remarkable*). There is an exponential decrease in the ratio of the use of type 1 words to type 2 words during the interval 1966–95, which requires some explanation.

There seems to be a general trend in the scientific community (among whom none is more guilty than the present correspondent) to elaborate the titles of scientific publications by the use of evocative words and phrases. Possibly the increased use of *reliable*, *secure*, *consistent* or *precise* reflects a willingness to concede to the anticipated preference of referees, who might be thought too wise to fall for such obvious self-aggrandisement as that

generated in an author by the use of *novel*, *new*, *exciting* or *remarkable*. However, at a time when the recruitment of the most able school-leavers into science is increasingly threatened, we should not perhaps go too far down the road of self-effacement, but try rather to convey the excitement of discovering that you really can make new life out of old rubber tyres.

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Alas, poor title

SIR — A systematic computer search of recently published papers in the biomedical sciences has confirmed what our everyday experience had led us to suspect: that authors and editors of review papers (such as appear in the News and Views section of this journal) are predisposed to use one of three catch-phrases in making their titles. In the past five years, the phrase “A tale of two. . .” has found its way into no fewer than 61 titles. Variations of “Of mice and men” have been used 32 times, and “Much ado about. . .” a respectable 22 times. (Eight per cent of the offending titles were in *Nature*.) We call for an immediate moratorium on the use of these hackneyed titles. Surely other works of English literature with titles amenable to such pillage can be found.

If something is not done soon, we fear this trend will reach its logical conclusion with “Much ado about a tale of two mice and men”.

Anne Moon

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Bright spark?

SIR — Max Delbrück, were he alive today, would be amused to find himself described as an electrical engineer (“Gaps in molecular biology” *Nature* 372, 33; 1994). He initially studied astronomy, and completed a doctorate in theoretical physics, with Max Born as major professor (E. P. Fischer & C. Lipson, *Thinking About Science, Max Delbrück and the Origins of Molecular Biology*; Norton & Co., New York and London, 1988).

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Randomization

SIR — In the Commentary article “Making medicine more scientific”, Hiatt and Goldman suggest that “new statistical approaches” might somehow be able to rescue from computerized hospital records reliable information as to which treatment strategies produce the best long-term outcome for the patient¹. If true, this would be wonderful: after all, a computerized analysis of data that already exist would be much easier than a randomized trial.

But it is not true. The reason is that where there is important uncertainty as to which of two treatment strategies to prefer, the real difference in long-term outcome might well be negligibly small, or it might well be of moderate (but humanly worthwhile) size, but it is unlikely to be large. So clinical research needs to be able to discriminate reliably between zero effects and moderate effects, and it cannot do this if it adopts methods that are potentially subject to moderate bias, or to moderate random error.

Clinical research must therefore use methods that guarantee that any biases will be negligibly small (which, in practice, can usually be achieved only by proper randomization, followed by appropriate statistical analysis), and that guarantee that the play of chance will likewise be small (which usually requires large numbers of patients to have been studied). Non-randomized methods, such as those now being introduced at great expense in the United States, cannot exclude moderate biases and so cannot yield reliable comparisons between plausible alternative treatment strategies.

Of course, randomization is not needed to show that prolonged cigarette use is a cause of most deaths from squamous cell lung cancer, for the excess among habitual cigarette smokers is so extreme². But large-scale randomized evidence³ is certainly needed to determine whether or not today's chemotherapy can cure some of these patients, for the proportion cured is not large. If Hiatt and Goldman want medical education to help make medicine more scientific, then they should not mislead themselves or their students into supposing that “results approximating those from trials” can be “extracted from routine clinical data”.

Richard Peto

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1. Hiatt, H. & Goldman, L. *Nature* 371, 100 (1994).

2. Peto, R., et al. *Mortality from Smoking in Developed Countries 1950–2000* (Oxford University Press, Oxford, 1994).

3. Peto, R., Collins, R., Gray, R. *Ann. N.Y. Acad. Sci.* 703, 314–340 (1993).

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Training for Africans in Africa

SIR — You suggest (*Nature* 371, 269; 1994) that the Republic of South Africa should do more to train young Africans in the techniques of palaeoanthropology.

This department at the University of Witwatersrand has, under Raymond Dart's leadership (1923–59) and then under Phillip Tobias's (1959–90), been recognized as an international centre of expertise in palaeoanthropology.

It was in this department that Dart removed the 'Taung child' (*Australopithecus africanus*) from its breccia, and Tobias and his colleagues have made remarkable early hominid discoveries at Sterkfontein, Makapansgat and Gladysvale. During this period, the teaching of human evolution was prohibited within the South African public educational curricula, but the tradition of palaeoanthropological research within a South African context continued, and is continuing unabated, as the 'new South Africa' takes shape under President Nelson Mandela's leadership. Undergraduate science courses here have, for 75 years, taught palaeoanthropology to South African students and these have recently been revamped to encourage the development of African postgraduate research students.

The department's Palaeoanthropology Research Unit (PARU) houses one of the world's largest collections of early hominid fossil remains, and these are continuously being visited and studied by the world's leading palaeoanthropologists.

A new, South African based non-profit organization, the Palaeo-Anthropological Scientific Trust (PAST), has recently been established to promote palaeoanthropological research in southern Africa having as its expressed mission the education of Africans about their prehistoric past.

The trust is unique in being based in Africa and in being supported financially primarily by South Africans. The education mission will be achieved through educational programmes, scholarships and the promotion of opportunities for African students, and students from throughout the world, to learn the skills required to become palaeoanthropologists of the highest international quality.

In 1995 the first 'palaeoanthropological summer school', designed to give training in the scientific methods required for palaeoanthropological research in southern Africa, will be initiated at the University of Witwatersrand with the financial support of PAST.

Budgeting has been so constructed that suitably qualified African applicants will be able to attend virtually free of charge. In this way the "hope" for free-standing expertise in the sub-Saharan centres is being realized and, perhaps most impor-

tantly, this initiative comes from the people of Africa.

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Population control not the answer

SIR — Two recent leading articles in *Nature* (370, 583–584 & 371, 185; 1994) regarding the Third International Conference on Population and Development deserve much thought and comment. The very idea that political institutions such as the United Nations have either the right or the ability to direct demographic transitions related to population control is not only pretentious but also unsupported by any historical precedent. Far from providing increased levels of freedom for all, or specifically providing increased "empowerment for women", any sort of population control statement can serve only to imprison people (in particular women) in a place where freedom is all but a distant memory. Population policies such as those proposed at Cairo serve to legitimize practices such as those in China where women are not only allowed to have abortions, but are required to do so if they already have a child.

The first leading article suggests that it is the presence of "uncivil inequalities between rich and poor that mar too many people's lives" that is really the issue at stake, not hysterical fears that the planet cannot support in excess of 7 billion inhabitants. Clearly, the writer has ignored the more important issue of the struggle for political power as a major contributor to "marred lives". Some of the most inegalitarian and underdeveloped nations, those at which the conference's statements are primarily aimed, have no need of population control policies at all; the wholesale slaughter of more than 100,000 in Rwanda and the use of food as a political weapon in Somalia are ample evidence of this.

The basic assumptions underlying the United Nations' desire to orchestrate improved living conditions and world harmony through population control are noble, and even plausible at first glance. Upon closer examination, it becomes clear that population control is not the answer because it does not address the underlying root of unrest and inequality in the world. For all of recorded history, man

has been cruel to his fellow man in order to serve his own desires; this has been a constant regardless of world population or demographics. To think that man can by himself solve these problems with statements of policy and forced "moral" (that is, the new morality of liberalism) behaviour is the utmost in arrogance.

Thomas A. Blcsak

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Telescope site at Mount Graham

SIR — The Commentary (*Nature* 372, 215–216; 1994) by University of Arizona astronomers ignores the views of Native Americans about our Mother Earth. To us Apaches, Dził Nchaa Si An (Mount Graham) is a most sacred mountain. It is the home of the *Ga'an* or mountain spirits. The *Ga'an* give the Apache people guidance, direction, knowledge and healing. Since time immemorial we have gathered special plants and herbs there. Many of our dead are buried there. Our elders and Medicinepeople have signed petitions opposing this desecration. Our tribal council informed the university of our opposition in December 1989. Our council reaffirmed its opposition on 10 July 1990. The university, the Vatican Observatory and the Max Planck Institute ignored our pleas and protests. They levelled the trees at the summit telescope sites on 2 October 1990. Subsequent council objections in June 1991, December 1992, May 1993 and as recently as October 1994 have also been ignored.

Finally, when we sued them, the court said we did not give them enough advance notice! Astonishingly, the astronomers argued in court that their project was exempt from all laws protecting Native American cultural and religious values plus all environmental laws as a result of a rider they had spent millions of dollars lobbying through Congress. The court refused to consider the long-standing scholarly proof in the university's own library, or the affidavits of our Medicinepeople and elders about the mountain's sacredness.

As studies by White astronomers show superior astronomy sites both in the continental United States, Hawaii and Chile, it is difficult to understand why 'enlightened' educated institutions are still, today, warring against our beliefs.

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Science for art's sake

Sukumar Vijayaraghavan

Hollywood's absent-minded professor, always a cliché, is now more unrealistic than ever before. Just how can biological research move forward in the modern era?

HAS 'big picture' science become extinct? Have we carried scientific methodology to a point where we stifle creativity? Is scientific research doomed to expand laterally, and has progress in scientific knowledge become merely a probabilistic event determined more by serendipity and numbers rather than the genius of the individual scientist? Today's biologist is more likely to dream of receiving a pink sheet that puts his or her \$300,000 RO1 grant in the top 14 percentile than to fantasise about wonderful groundbreaking experiments. At a time when billions of dollars are being spent to appease the appetite of the huge industry of the scientific enterprise, it is surely appropriate to evaluate, once again, our definitions of 'good' science.

Advancement in our knowledge of a field seems to come in tiny bursts after enormous investment of time and money and hundreds of papers, many of very little consequence. The goals of grand projects inevitably become more modest, even though applications are still phrased in terms of global advances to obtain funding. I am not arguing against examining the role of an amino acid in altering the desensitization rates of a receptor, but rather for doing so in the process of attaining a bigger goal. My plea is for science to be conducted in a more creative and integrative fashion.

Many of us would blame the ever-tightening financial situation for proliferation of mediocrity, perhaps rightly so. At a time when the number of grants being submitted has escalated exponentially, the money allocated for research has not. It is, therefore, not surprising that grants funded are those that are the most likely to work and that are backed up by preliminary data. There is a growing feeling that unless the results are knowable in advance, funding agencies will reject a proposal. It is, therefore, not uncommon for a principal investigator to spend most of the time and money allocated for a project on obtaining preliminary results that will ensure the funding of the next grant. Definitely not a prescription for creativity.

That, however, is not the only ailment that cripples the beast. As science has become more specialized and irrevocably separated from art and philosophy, the scientist has become more reductionist and isolated. From a time when science consisted of biology, physics and chemistry to one when we divide neurobiology

into molecular, cellular, systems and behavioural neuroscience, the individual scientist now knows more and more about less and less. Today's cellular neurobiologist knows practically nothing about the work of his or her colleagues studying behaviour and vice versa. Scientific method, as commonly defined today, is reductionistic: very little effort is devoted

integral part of the scientific process.

A third problem has to do with the hierarchical structure of science. Research has always had a whiff of élitism to it, a 'country club' mentality. Pedigree is invariably important in an individual's advancement, whether it is in terms of obtaining jobs and grants or of publishing papers in major journals. It is assumed that good laboratories produce good students and postdocs, though one would logically have to assume that a researcher from a minor university in small-town Mississippi who publishes a paper in a first-rate journal must, by necessity, be more creative than his or her counterpart in Harvard (for example), whose supervisor churns out ten such papers annually. The obvious effect this has on the careers of scientists who have failed to establish an acceptable pedigree is, of course, an issue for a separate debate. But this has also a way of stifling the progress of science. More and more universities are being staffed with postdocs from a few big-name mentors, carrying with them similar approaches and attitudes.

We need to bring back artistry and creativity to science. This does not necessarily have to be at the expense of scientific methodology. There are numerous intermediates between the dreamy and unrealistic and the emotionless and logical clichés of science fiction. Every scientist needs to have his or her perspectives broadened, and the way to do this is obvious: more interaction among disciplines. I propose that all research institutions have an open forum, a club whose membership consists of anyone interested in science. Members can then discuss their interests with physicists, chemists, biologists, economists — even poets.

A key feature of the discussions will be their lack of formal structure. Talks will consist of concepts and implications rather than experiments and controls, and any member of the audience is invited to interject with his or her own perspective. Ideas will be restricted only by logic and not by feasibility. At best, such a communal free-association will result in the development of novel ways of looking at scientific problems, and at worst scientists will receive perspectives from outside the ivory tower. □

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Eureka — origin of a citation classic?

to integrating different levels of a problem. As a result there is a massive lateral expansion in our knowledge, with little to show in the way of new concepts and innovations. The average scientist is a lot more adept at designing adequate controls for an experiment than at developing a new approach to solve a problem. There is a tremendous reluctance not just to be speculative in discussing one's work but even to extend the discussion to the next logical step. Surprisingly, this is true even in review articles, most of which are nothing but compilations of work done in a particular area. I believe that we have made scientific criteria for the interpretation of data so stringent as to eliminate the inspiration and idealism that once were an

What next for science in Central Europe?

This survey of science in Central Europe, the first of two, raises questions about the degree to which the planners of the new regimes should be constrained by the habits of the old. More radicalism would be welcome.

WHAT is to happen to Central Europe, and to science there? The pages that follow are an attempt at a survey of the present state of research and higher education in five of the countries of this important tract of the European land-mass. The survey follows by four years an earlier study of this kind (*Nature* 344, 599–620; 1990), which was itself close on the heels of the cataclysm of 1989, the year in which what was then the Soviet Union elected to withdraw its political and military influence from the most ancient parts of Europe. Inevitably, much of what follows describes the adjustments the governments concerned are seeking to make to the new opportunities, and the new hazards, facing them. It is an account of a region still in transition.

As always, it is important that the unavoidable defects of a survey such as this should be clearly understood. First, it is a snapshot: an account of where things stand now, not of how they became like this or of what they will be. Second, it is incomplete. On this occasion, nothing is said about Hungary, probably the state in Central Europe with the greatest scientific potential — except that the cover of this issue, a photograph of the Danube at Budapest, should be a reminder of Hungary's centrality. (A separate account of science in Hungary will appear in *Nature* early in 1995.) Nor are Slovenia (shaken loose from the former Yugoslavia at the end of 1992) and the three Baltic states dealt with separately; all of them, no doubt, have distinctive tales to tell. One of these days, Ukraine will seem a natural part of a survey such as this.

A further impediment to such surveys is that of giving fair weight to the extent of the social changes under way. Those who read what follows should consider this arithmetic. People born before the outbreak of the Second World War, which involved all the countries concerned, will now be 55 years or older. Those who recall Bucharest when it called itself the Paris of the East will be even older, making up a tiny fraction of the present population of Central Europe. And while the war was bad enough, the half century that followed was in many ways more debilitating of the spirit. Freedom (to travel, to study, to write, to converse) was not explicitly denied, but traded for complicity in illiberality. How is it possible to recover from such long anguish?

Depressing though the accounts that follow may be, they embody one consis-

tently cheerful message: Bulgarians still make jokes, the whole of Central Europe is seized of the importance of putting higher education and research on a sound footing and much has already been accomplished in that direction. The social transformation of Central Europe in the past five years has been more rapid than comparable transitions in the West. In Britain a few years ago, the outgoing Thatcher government could boast that it had returned to the private sector about a fifth of British industry that had previously been nationalized. In Central Europe, where all of industry and much of commerce were publicly owned at the beginning of 1989, there is now hardly a single country (Romania is the laggard) in which the contribution of private industry to the gross domestic product is less than a half. And the process continues apace.

How can that be squared with the hankering after the old days (1945–89) reported on several of the following pages? Easily, of course. The former Soviet Union was a worldwide Public Works Administration on the lines of that created in the United States as part of President Franklin D. Roosevelt's New Deal. Why else would Moscow have been paying Bulgarians to mine uranium when the material could have been had on world markets (or even from Armenia) at a tenth the price (see page 610)? Under that system, on admirable Leninist grounds, science and technology were particularly well favoured. (It is, of course, a great misfortune for everybody, that so little attention was paid to their effectiveness.) But what the hankerers in Central Europe overlook is that the old system has gone for good. Russia may yet take the place of the former Soviet Union as an assertive major power, but it will not repeat the old errors of the Eastern bloc. There is no way back.

What way forward may there be? That is evidently what concerns the leaders of the research profession in the five states covered by this survey. It is natural that they should be most concerned with the reform (to use the standard word) of institutions inherited from the past. How should the academy of sciences be organized? Are citation indices reliable guides to people's quality as researchers? Should foreign referees help to adjudicate research grant applications? What should be the balance of power between university rectors and their faculty deans? How should institutes conduct themselves

if their budgets cannot even cover their people's salaries? Who should pay for applied research, the government or the presumed beneficiaries of it, privatized industry? And what if such industry as there is has no interest?

Cruel though it may seem, all these questions are academic in the sense that they are irrelevant to immediate needs. That so much attention is paid to them in the emergent countries is a measure of how poorly appreciated is the extent of the social transition on which they embarked five years ago, and how far there is still to go. The difficulty is largely psychological, that of contemplating a future without the institutions made familiar in the past half century. Everywhere, for example, people are exercised by the status and function of the national academy of sciences, but Germany (no slouch in these affairs) has rubbed along without one since the end of the Second World War (and only now seems inclined to fill the gap — see *Nature* 372, 393; 1994).

The sad truth is that social and economic reconstruction on the scale now being attempted in Central Europe cannot but play havoc with the power of national budgets to support research on a scale commensurate with publicly sponsored research under the old regimes. Indeed, research in Central Europe cannot hope to prosper until there has been substantially more progress towards social and economic reconstruction. A spell of marking time seems inevitable. But that does not imply that there is nothing to be done. Indeed, the truth is quite the opposite.

Three goals must have priority for the years immediately ahead, one of which must be to ensure that the present strengths of Central Europe are not lost to the attrition of economic deprivation. This swathe of land may not claim to be the cradle of civilization (Greece does that), but it might. Remember Copernicus, and then Berthold Brecht's "Mother Courage". In modern times (and long before the Second World War), Central Europe has been a citadel of learning and literacy. It enjoys that benefit still. The hope must be that the social benefits of good schooling will not be put in hazard by the turbulence that inevitably lies ahead. Researchers anxious to safeguard the future reputation of national research should worry first about secondary education, the most fragile link in the scientific enterprise. "Go teach school, then!" is not an impolite remark to an academic

complaining that there is nothing else to do.

Second, Central Europe must put its universities in good shape. They include, of course, many of the world's most revered institutions, still-living proofs that the Middle Ages were not that dark. A century ago, Prague and Kraków were on mainstream Europe's academic beat. (Ernst Mach, Einstein's inspiration, taught at the Charles University, Prague, for many years.) But the Soviet period was disastrous for the universities of Central Europe. By separating research from teaching, the fallen regimes arranged not only that teaching would be less compelling than it might be, but that the institutions responsible would ossify.

Those in Central Europe who now break their heads on the organization of academies and their institutes would therefore be better occupied in reintegrating research with higher education. It is a simple but well established truth that students are taught best by people who have learned not merely what problems can be solved (and how), but who have at the centres of their minds an awareness of problems that nobody has yet tackled successfully. Even researchers who have not (or cannot) solve their chosen problems have a lot to say to students. Central Europe should be eager to give them a chance.

There remains research, both basic and applied (in an old nomenclature). Throughout Central Europe, there seem to be conscientious administrators worrying about the scale on which research should be supported, and the fields in which it should be directed. Both anxieties are misplaced. Simpler questions are more appropriate. In fundamental science, the issue is simply whether a person or a group is engaged on work that has contributed and will contribute significantly to our understanding of the world. If so, that person (or group) should be supported generously, but not so as to purchase the understanding that will follow. The purpose is primarily to encourage others who see the benefits of having the scales fall from in front of the eyes, but is also to enliven the institutions in which the work is done. It is unthinkable that wise choices can be made on such important matters without advice sought internationally.

Applied research is a different matter. On the Soviet model, applied research was variously the servant of industry and its lodestone. Considerations of economy seemed to compel the concentration of applied research in dedicated institutes. Only lately has it been generally accepted that the seeming economy is not an economy at all, but a recipe for arranging that researchers tackle problems in which industry has no interest and that industry must get by without essential technical



help. Central Europe must therefore say to itself that, one day, it will be as prosperous as Western Europe, but that it cannot yet know how that will be done. (Adam Smith's invisible hand will eventually provide the answer.) So, meanwhile, it should find ways, perhaps through taxes, to encourage industry to support its own research. That, late in the day, is what Western Europe is attempting. Why not, in this respect, short-circuit the West?

There remains the question of how the West can help. Mere self-interest dictates that it should, and more vigorously than in the past. To many, political considerations will be paramount; hitching Central Europe to Adam Smith's wagon will prevent a recurrence of the past half-century's desuetude. That is not a particularly worthy cause. Much more important is that a broadening of the European community of scholarship, in a sense not necessarily linked with Brussels, will benefit scholarship everywhere. It goes without saying that the inheritors of Hungarians such as Eötvös, Gamow and Teller may have as much to contribute to understanding as their compatriots.

Practically, there are many things to do. The European Union's fund for assistance in Central Europe has been the most significant influence in recent years (see page 600). It should continue, but should be changed. It does not make sense that support for research in Central Europe should revolve around tripartite (or even multi-party) collaborations. If a potential partner in Central Europe commands the

respect of a respectable partner in the West, that should be sufficient. And while people have recently been concerned to emphasize the importance of letting people from Central Europe travel to the West, it may now be more important to find Western academics willing to teach in Central Europe. The next Ernst Mach will be worth his weight in gold.

Of the institutions in the West that need to change, the European Science Foundation (ESF) is the most obvious. A week ago, this excellent organization was asking in public what its continuing link should be with the European Union. One difficulty is that it seems to have been upstaged in that regard by more recently created groupings, consortia of national grant-making agencies, for example. But ESF's claim on public attention has always been that it is a catholic organization, willing to take in members from where they wish to belong. Would it not make sense that, for an interim period, ESF should be Western Europe's channel for assistance towards the East? □

Science in Central Europe

This survey has been compiled as follows: Poland (pp 593–597), by David Dickson, News Editor, *Nature*; Slovakia (pp 598–600), and the Czech Republic (pp 601–604) by Allison Abbott, Chief European Correspondent, *Nature*, Munich; and Bulgaria (pp 608–610) by Carl Levitin, Head of Nature Moscow Bureau.

Polish science stands at the crossroads

Warsaw and Kraków. Seated round a large centuries-old table in Kraków's Jagiellonian University, a group of faculty members nod vigorously. The main dilemma facing modern Poland, they agree, is to decide what type of industrialized economy it aims to be. Meanwhile, attempts to create a modern science policy are arguments in a vacuum.

Five years after the fall of Communism, the dilemma is as sharp as ever. Much of the initial euphoria has long evaporated, as rapid inflation and stringent economies have combined to cut ever more deeply into research and teaching budgets.

So, too, has confidence in the effectiveness of 'quick fix' solutions, such as removing responsibility from the Polish Academy of Sciences (PAN) for the budgets of its 70 research institutes, transferring it to a new State Committee for Scientific Research (KBN).

Furthermore, the opportunities for exploitation opened up by the slackening of state control, combined with decades of experience in pitting wits against a widely despised political system, have given rise to new forms of corruption in science, just as in the rest of the economy. "I spend 40 per cent of my time teaching, 20 per cent of my time doing research, and 40 per cent of my time earning money," says one lecturer at the University of Warsaw, who says that he and his colleagues all take second jobs to earn a decent wage.

Optimism among the reformers, many of whom developed their ideas during the dark days of military rule in the 1980s,

nevertheless remains high. They hope that if the economy continues to grow at its present rate, removed threats of a counter-revolution will gradually recede.

Witold Karczewski, chairman of KBN and thus de facto minister of science, for example, admits that next year's science budget will remain the same relatively low proportion of total government spending (0.56 per cent) as this year. But he points out that this is based on an assumption that the economy will continue to grow, and that the government's pledge to raise spending by 5.5 per cent over the forecast rate of inflation therefore promises "some improvement in real money".

Polish science — like much of that elsewhere in Eastern Europe — has had a chequered history since, under Soviet direction, it was formally adopted as a state priority with the creation of the Polish Academy of Sciences in 1953. Academy researchers enjoyed a measure of intellectual freedom and many branches of basic science flourished, chemistry and mathematics in particular.

This success is reflected in citation figures collated by the Institute for Scientific Information in Philadelphia. According to a study commissioned by KBN and published in June, during the 1980s Poland was consistently ahead of comparable east European economies in the number of cited publications, as well as in the overall proportion of publications that were subsequently cited.

Indeed, taken globally, Poland came 11th in the world in terms of total publica-

tions in chemistry in the period 1981–92, 12th in physics and 14th in engineering, material science and mathematics. Many are proud of this record. "We feel that the old system gave good results," says Adam Bielański, vice-president of the Polish Academy of Sciences.

But the figures are deceptive. They fail to reveal a basic flaw in the Polish research system — the ineffectiveness, despite the exhortations of Stalin and his successors, of links between the activities of scientists and the application of their work to industrial and social needs.



Bielański: defends achievement record.

Many believe that, for the communist system, the flaw was fatal. "It was always promised to the state that support for research would result in the growth of the national economy through new inventions; usually it was not fulfilled, and it all broke down in confrontation with Western technology," says Zbigniew Grabowski, at the academy's Institute of Physical Chemistry in Warsaw, president of the Society for the Advancement of Arts and Sciences.

But five years after the fall of communism, the gap remains as wide — though now for different reasons. "Western industry is not interested in research carried out in Poland, while Polish industry has no long-term plans, and is therefore reluctant to invest in research," says Piotr Petelencz, professor of chemistry at the Jagiellonian University in Krakow. "In such a situation, there is little prospect of a substantial influx of research money for five or ten years to come."

Inevitably, government moves to reduce state spending and, at the same time, to enforce accountability for the spending of limited funds, have generated resentment. The critics' targets are the government's calls for greater selectivity in funding, and for the increased use of quantitative indicators — such as citation analyses — to assess effectiveness.

But many support these developments. "The scientific community in Poland has to be prepared to restructure itself and to change its values," says Andrzej Ziabicki, a prominent polymer scientist who is vice-president of the Stefan Batory Foundation, which has recently been using funds provided by the Hungarian-born financier George Soros to stimulate interest in the assessment of research. "There is no concept of science policy in this country; peo-

Historical figure hits the high notes

The face of Nicolaus Copernicus, the sixteenth-century astronomer educated in Kraków, looks out enigmatically from Poland's 1,000 złoty note. In former times, this would have had a significant value. Today, after several years of rampant inflation, the exchange rate stands at 22,000 złoty to the dollar — which values the note bearing Copernicus's portrait at about five US cents.



The difficulties faced by tourists and locals alike in counting the zeros, whether on bus tickets or hotel bills (the latter now measured in millions), will disappear next year, when four zeros are knocked off the currency. This will bring the value of the złoty roughly in line with that of its Western neighbour, the German Deutsche mark.

In turn, the złoty will be divided into 100 'groszy' — as in Austria (which used to occupy the southern part of the country as part of the Austrian-Hungarian empire). Copernicus's fate is uncertain, but he is unlikely to vanish completely from the currency.

Less fortunate is likely to be the Communist general Karol Świerczewski, who helped to rid the country of the Nazis at the end of the Second World War. His face still appears on the 50-złoty note. But with a value of less than a quarter of a US cent, that has already virtually disappeared from circulation. □

ple do not understand that science policy is about money and about priorities."

There has been some attempt to move in that direction. KBN, for example, has already carried out three separate exercises ranking the competence of the research institutes it funds. Similarly, its new research grants procedure (see below)

requires a detailed accounting of the likely outcome of proposed projects.

But each of these procedures, scientists claim, is open to corruption. In the grading of institutes, for example, relatively few have been given marks so low that their existence would have been threatened; most continue as before. Similarly,

there are arguments that the anonymous refereeing of grant applications is impossible in a relatively small scientific community, where experts in any field tend to be well known to each other.

Too often, critics claim, the result is an extension of the cronyism that used to flourish. "Science used to be a feudal system; the system has now been changed into an early capitalist system — but everyone is now corrupt," complains one university scientist.

In the circumstances, some argue that the positive aspects of the previous system may disappear if the transition to the new is too rapid. "The grants system, for example, risks leading to a disintegration of the scientific community, even at the level of individual institutes," says Edmund Przegaliński, director of the academy's Institute of Pharmacology in Kraków.

Others go further, claiming that the whole pace of change in Polish science has been too rapid. "I feel that the changes have been too big and too fast," says Henryk Frąckiewicz, director of the academy's Institute of Fundamental Technological Research (and formerly scientific secretary of the academy itself). "Only steady evolution can create a positive effect."

Such views, building on wider discontents in the scientific community, have helped to fuel the arguments of conservative critics in political circles. At least one prominent politician, for example, has recently demanded the abolition of the KBN, and the re-establishing the authority of the academy of sciences as Poland's main research funding body.

But supporters of reform claim that the problem lies elsewhere, namely in the refusal both of the government and of Polish industry to give sufficiently high priority to a strong national research base — including the need to train and support young researchers.

To support their case, they point to the low salaries of university faculty members which, at about 4 million zloty (US\$200) a month, is about the same as that of cleaning staff in government buildings — and can be as little as a third or a quarter of what new graduates earn as managers in industry.

The situation has historical roots. "In contrast with the former Soviet Union, science has never been well-paid in Poland," says Ziabicki. It also has many consequences. Many graduate students can often continue their studies only with financial support from their families. Further, many scientists take second jobs; one research director in Warsaw says that he is reluctant to fire a member of his group who turns up to work for only two hours each day, since it would be impossible to find a replacement.

To some, the solution is obvious: a 'rationalization' of the scientific community, both in universities and academy

A crusading reformer with a mission

Warsaw. Ten years ago, Witold Karczewski, then professor of neurophysiology at the Polish Academy of Sciences, was a leading member of a group of reformist scientists, all members of Solidarity, who prepared a set of proposals for the radical overhaul of Polish science.

Now, Karczewski not only heads the organization set up in 1990 to put such reforms into effect — the State Committee for Scientific Research (KBN) — but is also the longest surviving member of a cabinet whose political make-up has changed sharply since the communists were ejected from power in 1989.

"They call me the dean of the cabinet," says Karczewski, claiming that one of the reasons for his survival has been his determination to keep science above political turmoil. "We are an apolitical ministry," he says. "That's why I am still here."

Many of the reforms that Karczewski has led over the past five years have been warmly applauded by the scientific community. He takes particular pride in Poland's move away from the institutional funding of science through the Polish Academy of Sciences — previously responsible for the country's basic science — to a new research grant system.

"For the first time in the history of Polish science, we have a system of peer-reviewed grants," says Karczewski. "We have begun to break up the old feudal system of distributing research funds; for example, the new system allows young scientists to apply for their own research money."

Karczewski is also pleased at the evolution of the grant system towards greater selectivity. "At the beginning, my colleagues tended to give out as many grants as possible, and the success rate was far too high; this was totally wrong, and we are now turning the screw."

But Karczewski has been less successful in obtaining a significant increase in funding for research. Under the communist regime — as his critics frequently point out — research took around 1.5 per cent of the gross national product; this year it has dropped to 0.56 per cent of government spending, and, despite broad

promises from the prime minister, it is likely to stay at the same level in 1995.

Karczewski acknowledges that success in winning more support for Polish science will depend on whether it can be linked to broader goals endorsed by its country's industrial and political leaders. But he points out that a policy statement issued recently by the Minister of Finance, Gregori Kołodko, on the need to invest in human capital, includes a clause that says that the decline in Polish education and science must be stopped. "I have a weapon that I can use," he says.

But Karczewski is also aware that the difficulties experienced by Polish scientists (low salaries, lack of research funds) have given support to those who argue they were better off under the communist regime. "Some people would be willing to go back to the old, safe system," he says. "I find that unbelievable."

Yet even KBN is not blameless, even to reformers. Centralism persists. Under the 1990 reforms, all money for the laboratories run by government departments is now channelled through KBN. "Some people say that this is too centralized," Karczewski says. "During this transition period, tighter central control is unpleasant, but it is necessary."

Budgetary and political pressures on KBN have led Karczewski to make compromises which — like the broader policies of successive governments — have caused resentment among those impatient for more radical changes.

Despite earlier promises, for example, there has not yet been a significant weeding-out of less productive research groups or government laboratories.

After almost five years of juggling pressures for radical reform with the conservatism of the traditional communist structures responsible for science, Karczewski admits candidly that "it is a very hard job".

But he also says that he is "not willing to betray my fellow scientists". The feeling of support is reciprocal; whatever criticisms there are of the details of KBN's policies, many Polish scientists agree with one medical researcher that "he is the right man in the right job". **D.D.**



Karczewski: pride in progress of reforms

Academy rides the winds of change

Warsaw. Perched on the 26th floor of Warsaw's Palace of Science and Culture — a gift of Josef Stalin to the people of Poland — the Polish Academy of Science is struggling to retain its status as the country's leading scientific institution.

So far, despite a decision by the government in 1990 to transfer responsibility for the funding of its 77 institutes to the State Committee for Scientific Research (KBN), it has survived remarkably intact.

Unlike its counterparts in some other former communist states in eastern Europe, relatively few of its institutes have been closed. Nor have there been widespread redundancies among research staff (although many laboratories have cut back on technical assistance).

Adam Bielański, one of its three vice-presidents (and also president of its Kraków branch) points out that, in a recent grading exercise carried out by the KBN, almost 70 per cent of the institutes came out in the top 'A' category, qualifying them for a continued high level of support from the government.

Others point out that the academy, which has 350 full and corresponding members (as well as 190 foreign members), continues to play a crucial role in maintaining the collective identity of the Polish scientific community and stimulating interdisciplinary collaboration.

But the academy remains under pressure from critics who claim that its bureaucratic structures are outdated and heavy in their demands on scarce financial resources, and that the academy is insufficiently flexible in its scientific division of labour to meet the needs of modern industry.

Another complaint, frequently heard in political circles, is that the divorce of



Symbolic left-over: Warsaw's Palace of Science and Culture.

research from teaching — one of the most damaging legacies of the communist era — means that academy institutes play a relatively small role in meeting one of the country's main needs, namely the production of a new generation of scientifically trained university graduates.

Many academy scientists are themselves concerned about this situation. A group from two physics institutes and the institute of mathematics in Warsaw, for example, last year set up an independent 'College of Science' aimed at giving students a broad scientific training.

Teaching at the college takes place in the laboratories of participating institutes,

and covers basic mathematics, physics and chemistry for the first two years, leading to the equivalent of a US bachelor's degree at the end of the third. "Trained this way, students should understand the foundations of technology in many areas," says Kazimierz Rzazewski of the Centre of Theoretical Physics in Warsaw.

Spurred by such initiatives, the academy itself is now discussing plans for a more ambitious scheme to create what some refer to as a 'university of science'. It is actively encouraged by the Minister of Education who, says Bielański, "has said that he is very interested in using the potential of the scientists in the academy for teaching".

Not surprisingly, such initiatives are viewed somewhat sceptically by many university scientists, who see it partly as an attempt by academy institutes to justify their continued existence, and partly as a way of recruiting future researchers.

"We do not need outside professors to come and teach," says one prominent physicist. "What is needed is a deep reform of Polish science, nominating the best institutes as state institutes that we know we need — but not continuing to support all of the rest."

More reforms are, indeed, on the way. The academy is completing discussion of a draft bill it hopes the government will accept and which would, among other things, provide its institutes for the first time with independent legal status.

How deep the reforms will be remains to be seen. Those (including some academy members) who continue to insist that, in many ways, Polish science was better off under the communists, still have powerful friends in political circles. The jury is still out on which way the academy — like the whole of Polish science — will eventually decide to evolve. □

institutes, that would allow those remaining to be paid competitive salaries. "We have institutes of the academy all over the country that are drinking money," says one scientist at the Jagiellonian University. "Some of the institutes are excellent, with top-class scientists; but there are too many with a low scientific output."

Others suggest that the scientific community itself needs to adopt a more rigorous approach to its internal review procedures. "At present there is no system for self-purification," says Anna Grabowska, of the Institute of Physical Science.

One proposed reform is a change to the current procedure by which a university faculty member can become a professor merely by successfully surmounting a set of academic hurdles.

KBN officials claim to be well aware of the drawbacks of many existing practices in the research community. Karczewski, for example, acknowledges that there is a growing 'generation gap' caused by insufficient recruits to scientific posts in univer-

sities and elsewhere to fill the vacancies that will arise as older people retire. But they fear that too rapid an implementation of radical reforms could tip the hand of those who would like to see the committee itself — and its work — abolished.

Even Karczewski admits that the key to the future lies in the extent to which the government — and its backers in the international financial community — can be convinced that structural changes in the links between researchers and the implementation of their results are likely to succeed.

Further changes are on the way. A new statement on innovation policy, recently approved by the government, even flirts with the idea of privatizing government-owned research establishments — a radical step, even for governments in the West. To quell anxiety, the statement emphasizes that the principles would be different from those for the privatization of the state's main assets in 1989.

For many, however, the future health of

Polish science depends primarily on the health of its economy. "At present, there are no strategic plans for developing Polish industry," complains Marek Szymoński, a physicist who is vice-rector for international relations at the Jagiellonian University.

Even senior government officials admit that Poland has not decided whether it is going to be a high-technology country — the exploding economies of the Pacific Rim are mentioned — or one whose economy is based more on service industries and modern agriculture.

This ambivalence is reflected in Poland's science; attempts two years ago to impose strategic directions on research policy have since been dropped, but the 'invisible hand' expected to drive research in directions of national need has yet to make itself felt. "Poland is at so many crossroads," says one visiting scientist. "This can be a great opportunity; the problem is, how do you know which way to move?"

David Dickson

Universities struggle to grow

Kraków. Founded in the fourteenth century, and proud of its history at one of Europe's oldest seats of learning, the Jagiellonian University in the ancient Polish capital of Kraków has long been used to surviving political turmoil.

When Germany invaded Poland at the outbreak of the Second World War, for example, many of its senior faculty members were herded off to concentration camps. When the communist regime took over in 1949, the university found itself severed from its ancient medical faculty, and subjected to harsh central planning.

Now the main threat facing the university lies in the difficulties facing the gov-



The 14th century Collegium Maius (Greater College) of the Jagiellonian University.

ernment as it seeks to recover from the communist era without the resources to operate a modern industrial economy along Western lines.

"The deterioration in higher education in Poland is incredible," says Aleksander

Koj, an eminent molecular biologist currently occupying a second term as the university's rector. "The present higher education budget is about 0.7 per cent of the gross domestic product. This is far below other European countries; even in Hungary or Czech Republic they spend between one and two per cent."

As elsewhere in Poland, a constantly heard complaint is that the government is seeking a major expansion in the number of university students but has not provided the resources for doing this. "In the past four years, the number of students has increased by 50 to 80 per cent," says Koj. "At the same time, rapid inflation means that the higher education budget has decreased by 40 per cent in real terms."

The new regime has brought its benefits. Last year, for example, the medical school was reunited with the university for the first time since the 1950s. "The best aspect of this is that we have been able to regain a scientific stamp to our medical activities," says Ryszard Gryglewski, a former rector of the medical school who is now chairman of the university's department of pharmacology. "This means that we are not only medical professionals, but can rebuild our links with basic research."

Liberalization has given the university greater autonomy in handling its affairs. While some say this has made university heads hostage to the wishes of faculty members, others welcome greater internal democracy, and the scope is provided for academic initiative.

But government economy measures have taken their toll. The most common complaint is that funds for teaching are

insufficient. A university professor often earns less than graduates entering a first job — and some academics feel obliged to use scarce research funds to subsidize the purchase of teaching equipment.

Another concern is that, keen though it is to compensate for low university salaries by allowing them to be supplemented through research grants, the State Committee for Scientific Research (KBN) has also indicated that it will not pay for equipment costs in standard grant applications.

"These signals are rather worrying," says Tadeusz Sarna, head of the university's institute of molecular biology. "Those who want money to buy equipment are now expected to apply for special equipment grants through resource centres, as in the West. The idea is sound; but the scale here is very different."

Continuing tension between university science faculties and the institutes of the Polish Academy of Sciences does not help. The suspicion that the latter continue to receive privileged funding is strong, as is the concern that the academy institutes have done little to focus their efforts on the genuine needs of Polish industry.

There is also criticism of the government's algorithms for deciding financial support for individual universities. The calculations are based on the numbers of students and staff. One anomaly, pointed out by Krystyna Dyrek, a professor of chemistry, is that the effect of firing an inefficient member of staff is that "we get a reduction in our budget, so it is not worth it".

Like its counterparts in richer industrialized countries, the Jagiellonian University is about to embark on a major fund-raising exercise which, it hopes, will allow it to meet the challenges of the twenty-first century. One priority is a new institute for molecular biology; the current building, previously a Roman Catholic seminary, is being reconverted to its former role. The molecular biology institute may occupy a 'technology park' in the city outskirts.

Where it can, the university is also making use of funds from Western organizations to help the process of modernization. The Mellon Foundation, for example, has already provided a major endowment to equip the university with a high-speed computer link.

But Koj and others accept that the future will be secure only when the economy is sufficiently robust for the government to provide adequate investment in universities from its own resources. **D.D.**



Koj: Call for a boost to university funds.

Dream meets reality of Euro-funding

Warsaw. Despite political willingness on both sides of the former Iron Curtain to integrate Polish scientists into the European science community, progress has been slower than some had anticipated.

Some projects are proceeding well. Thus work on a number of infrastructure projects — for example, a high-speed data link between Warsaw and Stockholm — is already underway as part of the European Commission's PHARE programme, following a 1993 agreement between the EC and the KBN.

In addition, almost 50 Polish research teams are taking part in more than 30 research projects in field such as telecommunications, chemistry and agriculture, as a result of Poland's decision in 1991 to join both the European Science Foundation and the European Co-operation in Scientific and Technological Research (COST).

But many researchers have been disap-

pointed over the rejection of proposals submitted to other programmes funded through Brussels. "I have tried twice to apply for EC funds, each time jointly involving considerable work drawing up proposals with researchers from other countries, but both were rejected," complains one molecular biologist. "In the end, they did not even explain why our proposal was turned down."

Disillusionment is even creeping into official documents. A report prepared by KBN points out that although Polish researchers have been eligible, under a 1991 agreement, to participate in the EC's third framework programme on a case-by-case basis, "regrettably, so far no co-operation has been established on that basis". In particular, it says, Poland would be required to pay both the costs of the research and the EC's 'co-ordination costs' which "in Poland cannot be considered as trivial". □

Exploiting a medical tradition

Kraków. The astronomer Nicolaus Copernicus may be one of Kraków's (and Poland's) best known scientists. But, as an early graduate of the university town's medical college, he is also part of a long tradition of medical teaching whose reputation remains high — and which his successors are now keen to exploit.

Last year's decision to return the medical school to the Jagiellonian University, from which it was separated by the communists in 1950, has not only strengthened links between clinical medicine and basic research, but also helped to establish a platform from which to attract funds from foreign pharmaceutical industries.

Biomedical researchers in Kraków are exploring ways in which the specific conditions they are able to offer — in particular, high scientific standards combined with lower labour costs, and more liberal regulations on the use of animals in research — can be used to attract funds directly from Western drug companies.

Central to this strategy has been the creation of the semi-private Jagiellonian Medical Research Centre, an organization that links research groups in the university's science faculties, its medical faculty

and the Polish Academy of Sciences, Institute of Pharmacology.

One field of specialization is research on the cardiovascular system, the product of long-standing links with the pioneering work of Sir John Vane at St Bartholomew's Hospital in London which stem from an early visit by Vane to Poland. Linked to this is a research capability in inflammatory responses.

The centre offers the facilities of the university and the institute to companies wishing to study the biological activities of new compounds, quantify their therapeutic strength, and study how they work. To this end, it has already raised \$300,000, including a grant from the Japanese pharmaceutical company Yamanouchi.

"It is a way of raising extra funds to allow young people to do research," says Ryszard Gryglewski, the president of the centre, who was once a student of Vane, rose to become rector of the medical school and now chairs the university's department of pharmacology.

Edmund Przeglasiński, the director of the academy's pharmacology institute, says that his organization, which has had

long experience in working on the action of anti-depressant and other psychotropic drugs, now receives four to five per cent of its funding through contracts with Western drug companies.

"Costs are lower here than in other countries, while the level of research is not so different," says Przeglasiński. But, anxious to retain the centre's non-profit status, he emphasizes that he and his colleagues "work only on drugs of interest to us from a scientific point of view."

Jerzy Vetulani, the scientific director of the institute centre outlines its strategy. "We realize that it is difficult for us to be at the forefront of any one science," he says. "But we have the methodological diversity to look at several factors in any one animal, for example to investigate both the behavioural and physiological effects [of certain chemical compounds] and then study brain samples of the same animal."

Officially, both the government and the academy of sciences are keen to encourage such links between research groups and private companies. Indeed, one of the goals of reforms being drafted by the academy is to provide institutes with greater freedom to enter into such arrangements, the Kraków institute is already planning ways of dedicating some of its laboratory space to studies funded by foreign pharmaceutical companies.

Psychologically, considerable hurdles remain. "In Poland, money is not something that we like to talk about," says one scientist involved in such contract research. "There is an idea about the purity of science, and people are not proud of doing contract research for Western companies."

Nonetheless given the lack of Western interest in funding Polish research for its own sake — and Polish industry's inability to do so even, if it were inclined — surviving on contract research from the West is likely to become an increasingly important strategy for many laboratories. **D.D.**

Peer review: a call for help

Warsaw. "If the West really wants to help Polish science, it should spend less money and effort on travel grants and fellowships, and more on refereeing grant proposals from Polish scientists," says a senior academic from the University of Warsaw.

One of the major changes since the fall of communism has been the introduction of a fully fledged research grants system. For the first time, Polish scientists — including those working in the institutes of the Academy of Sciences — are having to submit research proposals to the scrutiny of their peers.

But it is widely acknowledged that the system has shortcomings and is also open to abuse. Even Witold Karczewski, the chairman of the State Committee for Scientific Research (KBN), admits that there is only one other scientist in Poland qualified to review his own grant proposals.

A vigorous dispute is now under way on the merits of reviewing grant proposals outside Poland. Advocates of this approach argue that it would provide a neutral buffer against social pressures and ensure that the quality of Polish research was judged by international standards.

"Why cannot we do what they do in the Czech Republic, where all grant applications have to be accompanied by a summary in English," says Anna Grabowska, a theoretical chemist at the academy's Institute of Physical Sciences in Warsaw.

But there are also opponents of the scheme, particularly, it is said, those who might be embarrassed by the comments of Western colleagues on the quality of their research, and who dress up their fears by claiming that grant proposals sent abroad risk being plagiarized.

As with other reforms, Karczewski is now moving cautiously. While encouraging scientists to send projects for foreign review, and describing fears of plagiarism as "ridiculous", he also admits that "I have to play it quietly."

"We could be successful if we gave top priority to those who agree to be reviewed abroad," says Karczewski. "But I do not want too many restrictive regulations."

Some are growing impatient, and criticize the staff of the KBN for their apparent unwillingness to require all grant proposals to include a summary in English, explaining their reluctance on a lack of Western contacts.

"KBN staff complain that they do not know the names of Western reviewers," says Adam Lomnicki, professor of environmental biology at the Jagiellonian University in Kraków, and a strong proponent of tough peer reviewing. "But if they do not know who are the experts in the West, there is something wrong with them; they are not real scientists."

Many realize that the foreign review of grant proposals is widely seen to be one of the quickest ways of helping Polish science pull itself up by its bootstraps, provided the right mechanisms — and political support — can be found. **D.D.**



Przeglasiński: 'costs less than elsewhere'.

Separate Slovakia's struggles

Bratislava & Nitra. Slovak universities have two big problems their Czech counterparts do not face. First, since Slovakia became independent of pre-existing Czechoslovakia in 1993, university staff have been leaving in droves. Second, the new law to modify the 1990 law of universities in federal Czechoslovakia, likely to be debated in parliament this month, will not make the radical changes expected in the Czech republic.

The 1990 act made faculty deans independent of their rectors, and so impeded attempts by universities to reorganize themselves along Western lines. Unlike their counterparts in the Czech Republic, Slovak rectors have failed to get these provisions changed.

Juraj Švec, rector of Bratislava's Comenius University, who, since 1990, has moonlighted as head of the Slovak Academy of Sciences Institute of Cancer Research, says that in 1990 it may have seemed sensible to spread power among the faculties. Academics were then worried by the risk of concentrating power in a single individual who, in an unstable political climate, could be subject to political influence, as in communist days.

In Švec's view, one unfortunate result has been to make university courses inflexible. Faculty deans may have been prepared to make changes to their curricula, but they were in general not prepared to coordinate their courses with those of other faculties. This has made it difficult for students to change courses, says Švec, and has also made universities expensive to run. When faculties duplicate courses, such as West European language courses and basic economics (now part of all study programmes), he says, "not only is it more expensive, but we do not have so much expertise in these areas, so that quality must be questioned."

In August last year, a group of 19 experts, selected by the ministry of education and headed by Švec, presented a proposal to put more decision-making powers in the hands of rectors to overcome this problem. But it was blocked by an outcry from the scientific community. The academic senate of Comenius University called for Švec's resignation, only withdrawing its demand when he promised that the proposal would be redrafted.

In the Czech Republic, the ministry of research was able to defuse a similar outcry. In Slovakia, academics were able to argue that their political situation continues to be unstable and that such a change could be dangerous.

Švec now diplomatically says that the academics' point is valid. But he points to other external factors which mean that universities must change if they are to serve the community.

Unemployment is a serious pressure, he says. In 1993, 0.5 per cent of the unemployed were graduates, but this proportion has now doubled. "This means that one-fifth of last year's 13,000 graduates did not get jobs", he says, arguing that this means universities, used as they are to being told by the Communist Party how many graduates to produce in each discipline, should now be flexible enough to adapt their courses to suit market needs.

In addition, the long-term desire of Slovakia to become a member of the European Union means that the country should adopt similar systems to those of Western Europe. "Universities are fundamental to European integration," says Švec.

But in Slovakia the changes are going to have to be achieved step by step. The new act does not go as far as many would like, but there are some improvements, such as the formal introduction of the PhD research degree, along Western European lines, and a formal 'habilitation' procedure. That is the German teaching and research qualification required of those wishing to be appointed to the 'Dozent', or assistant professor, level at a university. Habilitation was previously very much corrupted by politics: many so-called dozents, or 'telephone dozents', were appointed for political reasons.

But while arguments about the structure of universities now take centre stage in the academic community, a more serious problem is calling loudly from the wings: universities are losing their staff.

In the Czech Republic, despite low wages, negligible unemployment and

political pressure to cut staff on grounds of efficiency, there has been no reduction in staff numbers (see page 602).

In Slovakia, wages are not simply low, but improbably so. Academic salaries have lost 30 per cent of the value of Czech university wages since the countries split. Slovak academics are now almost the lowest-paid state-employed personell. At Comenius University, 1,400 staff, more than a quarter of the total, have left in the past 18 months. Of these, 880 were teaching staff. As a result of this extraordinary brain-drain, the average age of professors has risen to 62.5, and that of associate professors to 58.5. In a few years retirement will decimate the teaching staff.

Pressure for the formal evaluation of staff is also weaker. As in the Czech Republic, internal evaluation failed to satisfy critics from the Academy of Sciences and elsewhere (see page 601).

Ladislav Kabát, rector of Nitra's University of Agriculture, nevertheless argues that the critics have not given the universities credit for the particular problems they faced in the evaluation. As well as having to assess the competence of existing staff, for example, they were also required to reinstate all members of the academic staff who had been dismissed for political reasons in the previous 20 years, since the Prague spring. Kabát says that at his university, there were 100 people in this category, mostly over 60 and approaching retirement, whose competence had been lost in the intervening years.

Now, the urgent task is to improve pay. At the end of last month, rectors and trade unions produced a petition demanding an increase of salaries of 100 per cent for professors, 75 per cent for associate professors, 50 per cent for other qualified scientists and 20 per cent for all others.

The government has countered with an offer of a 10 per cent increase in salaries. "We will protest", says Švec. "We will stick to our request". Švec has more influence than most: he was became a member of parliament in the September elections.

Alison Abbott



Rector, administrator and parliamentarian.

Shrinking asset for funding

Bratislava. Science in the two republics of the former Czechoslovakia has suffered progressive reductions in funding since the revolution. Slovakia, economically the poorer partner, has inevitably fared the more poorly.

The Slovak Academy of Sciences has seen its budget more than halved, from SKr808 million in 1990 to SKr401 million in 1994. On top of this, the 1994 budget was recently been cut by a further 5 per cent as part of a general economy package. Over the same period, staff numbers

have been almost halved — from 6,220 to 3,340 — while cumulative inflation for the four years is some 100 per cent.

The unions of the academy have now, for the first time, begun a major lobbying campaign to restore government support. They are preparing a petition, already signed by the academy's presidium, to put their case to the government. A similar petition is being drawn up by the university unions.

Neither is likely to cut much ice. Like its counterpart in the Czech Republic, the

Slovak academy receives little moral support from government, which has little financial room for manoeuvre. Sympathy will probably diminish further when the current minister for education and research, Ľubomír Harach, who is popular with the academic community, is replaced when the new government is formed.

Tomáš Bleha, the academy's vice president, says that the sudden 5 per cent cut in October was an intolerable blow to an institution which, in the past two years, has been setting its house in order with fundamental reforms. Among other things, it has begun regular evaluations of research performance in its institutes by external committees made up of foreign scientists. Several have been closed. The academy's presidium has asked research institutes to maintain staff salaries, and to absorb budget cuts in their infrastructure budgets.

But these budgets cannot bear the strain, particularly because scientists are only poorly supported by the Slovak Grant Agency, their only national source of research grants. The agency was set up informally in 1992 as the Czechoslovak Grant Agency, with 14 expert committees to evaluate applications and with an annual budget of Kr450 million.

But by 1993, government resources were so scarce that there was no central contribution to the agency, and the academy and universities kept it going with a small part of their own budgets.

The universities and the academy continue to share the same committees, but money is not exchanged between them. This year, the Slovak academy was not able to contribute anything to top up the government's paltry offering of SKr31 million. This small sum was finally distributed fairly evenly between universities and academy institutes, presumably to avoid charges of favouritism.

Despite financial problems, the agency operates according to Western norms. Applications must be submitted in English so that they may be sent to foreign referees. But, in the circumstances, some scientists question whether this is really appropriate.

Anna Preťová, of the Institute of Plant Genetics, says that much of the time of foreign scientists is being taken up in assessing projects that simply will not be funded. One of her own applications was graded eleventh on the priority list but in the end only seven applications were selected for funding — and those incompletely. "It is embarrassing that our colleagues abroad know that their time is being wasted," she says.

Ivan Horváth, from the academy's foreign relations department, says that the passing of bills on the academy and on the Slovak Grant Agency, currently awaiting parliamentary time, would give both orga-

nizations legal status and set out clearly the terms under which they receive state funding. That, he believes, would solve most of the problems.

Whether such a development would help fundamentally is an open question. In any case, the bill has only low priority in a long queue of legislation, and must await the attention of a parliament preoccupied with its own internal fights.

Meanwhile, the academy's relationship with Slovak universities continues to be

strained. The universities have claimed the sole right to award the newly introduced PhD degrees. Before the revolution, the Candidate of Sciences research degree was also awarded by the academy, which feels that it has the appropriate experience to train young scientists. Under the new system, the academy will still continue its training activities, but only in collaboration with an accredited university. That, says Bleha, makes the academy very unhappy. **A.A.**

New plant in ancient soil

Nitra. "My institute was founded in the most difficult of times", says Anna Preťová, director of the five-year-old Slovak Academy of Sciences' Institute for Plant Genetics in Nitra, one of Europe's most ancient cities, 80 km east of Bratislava, off the road to Budapest.

She had not expected the political process of transformation of Czechoslovakia to be so detrimental to science, and she spends more of her time than she would like on lobbying politicians and the public for more support of science. Her institute, founded in 1990, is the youngest in the Slovak academy. The average age of its staff is the lowest — only 35. Its curious history is very much a product of Slovakia's turbulent recent past.

After Czechoslovakia's Prague spring in 1968, communist ideology adopted a principle of decentralization, to prevent concentration of political resistance. In line with this, the Slovak Academy of Sciences decided to move its biological institutes to a new centre at Nitra, where there was a strong agricultural university.

In the Czech Republic, there was a similar move in the early 1980s, when many scientific institutes were moved from Prague to České Budějovice in southern Bohemia, to a campus that also serves the Czech Republic's new university, the only one completely integrated with Czech academy research institutes.

In Slovakia, the move took a little longer, and a building intended to house all the biology institutes from Bratislava was begun only in the mid-1980s. In 1987, Preťová and her small group of plant geneticists, part of the Institute of Experimental Biology and Ecology, moved into temporary laboratories at Nitra, with the expectation that other departments and institutes would follow when the building was complete.

But the revolution intervened. After 1989, scientists at the academy's biological centre in Bratislava, who had always feared exile to Nitra and who believed

that the idea had been forced upon them by the communists, refused to carry through the plans to move.

Preťová's research group of 22 scientists, established in 1990 as an institute in its own right, was left to rattle around in the huge purpose-built laboratories when they were finally opened in 1992.

They have been rescued by another victim of the transformation. The Nitra-based Institute of Archaeology, which since 1953 had been housed in the Roman



Plant genetics — the plant genetics institute had some catching-up to do in the aftermath of Lysenko.

Catholic Church's majestic castle, was evicted at the beginning of this year, when the newly powerful church was able to reclaim its premises. Staff moved in to the new biology building, and the institute's extensive archaeological collection was placed in the Nitra Museum. The two institutes have also been joined by several private companies.

Preťová regrets that plans for a large multidisciplinary biology centre have not come to fruition, but is grateful that some sort of solution has been found. Regrets have in any case been overshadowed by the fundamental necessity of survival.

As with all academy institutes, Preťová's budget has been cut year by year. Last year, there was not enough in the coffers to pay all salaries. To avoid redundancies, the scientists agreed to accept 70 per cent of their entitlement, a principle with which the academy presidium was not entirely happy, she says.

This year, says Preťová, after months of lobbying, a compromise was reached. She dismissed the two of her staff who had the

lowest publication records (an independent institute must have a minimum of 20 staff) and the academy increased her budget by 15 per cent.

Even so, there is effectively no money for equipment. Because all scientists, from the academy and the universities are in the same boat, this has spawned a spirit of mutual support. Preťová has organized with the Agricultural University a shared molecular biology laboratory, in the institute building, which pools the resources of both partners. She has also struck a deal with one of the private companies in the building to use its amino acid sequencer.

Cooperation is not entirely pragmatic. Like many academy scientists, Preťová is keen to be involved in teaching the next generation of scientists. Her institute is creating a joint university-academy department for reproductive biology at the Agricultural University where many of her staff give lecture courses for token pay. She is also setting up a faculty of plant sciences in the local teacher-training college; money is available for a single post, and this is shared between Preťová, who will direct it, and two other institute scientists. Both will accept students in the next academic year.

Analogous to the struggles of the institute, the science of plant genetics in Slovakia has a history equally affected by politics. It was heavily influenced by the ideas of the Ukrainian plant physiologist, Trofim Lysenko, whose theory that genes can be modified by good husbandry was accepted as creed by the Communist Party, which in 1948 declared Mendelian genetics to be mistaken. Although the Lysenko influence had weakened by the 1960s, plant genetics never developed as a strong science.

Preťová has helped to bring standards of Slovak plant genetics to Western levels. She says she had a lucky break when she was allowed to attend a meeting in the Netherlands in 1984. To the envy of her coworkers, she was subsequently invited by an Australian scientist, who was interested in her work on plant embryogenesis, to spend six months as a senior scientist at the University of Melbourne. It took a year to arrange a visa, but it was worth the struggle, and the experience remains a formative influence.

In Melbourne, she learnt how to get plant embryos from somatic cells in culture, an advanced technique that she brought back to Slovakia. "I was amazed by how much time scientists dedicated to talking about their research", she says, recognizing that the interest in broadcasting their work was closely linked to the need to compete for research money. She was also amazed at the long hours scientists worked — "some even worked on Christmas Day" — but saddened to realize how handicapped her compatriots were by their poor knowledge of

Western languages.

Poverty is not an ideal breeding ground for scientific innovation, but Preťová's institute will survive, because of its success in establishing itself as the core of plant sciences in a country where the development of agriculture is fundamental to the economy. It will also survive because of the support of the many foreign scientists who want to collaborate, and because of the energy and determination of its staff.

But, as Preťová says, "just when one problem is circumvented, you are dealt another blow". This autumn, there were two: the withdrawal of 5 per cent of the institute's budget (in line with the finance ministry's decree in October that the promised budget would not materialize) and the failure of the embryonic Slovak Grant Agency for Science to fund projects that had been given the highest evaluations, her own included. **A.A.**

Looking separately to the West

Munich. Now that they are in charge of their own affairs, scientists in Central European countries do not spontaneously seek collaborations among themselves. Instead, they are looking firmly to the West.

The explanation is simple. Research standards in the West are generally high, and, more importantly, there is more money to support research. There are all sorts of permutations. Public grant-making bodies in the West support some collaboration, while there are also foundations such as Germany's Humboldt Foundation that support research. And networks of personal contacts increasingly span east and west.

Thus Václav Sklenička, director of the Institute of the Physics of Materials at Brno, has established a collaboration with the Kernforschungszentrum Karlsruhe (KFK), a national research centre in Germany, to develop new materials for use in nuclear power plants. There is no direct support from Germany, but the institute benefited from sophisticated stress-controlling and measuring equipment provided by the KFK.

But the most generous support has come from the European Union (EU), which over the past four years has offered a total of ECU262 million to support cooperative projects and networks, of which ECU110 million is allocated for this year.

That support is not part of the EU third framework programme, but is administered by separate programmes with names such as 'Copernicus'. But from the beginning of next year, cooperation with eastern Europe will become an intrinsic part of the fourth framework programme. Then there will be less money.

Only ECU220 million has been allocated to international cooperation for the four-year programme, while between a third and a half of what money there is will go to countries from the former Soviet Union.

But central eastern European countries will have the opportunity to participate, at their own cost, in all programmes of the fourth framework, including those related

to industrial research which up to now have been out of bounds. Since participation allows full access to research, EU officials believe the result will be a considerable advantage to the new countries.

The EU funds available for central eastern European countries next year will be spent mostly on joint research (projects and networks) in areas of common interest. The commission of the EU had identified environmental research as the most appropriate area, but the EU committee for international cooperation, meeting last month, was not happy with the details of the commission's plan, which now has to be redrafted. This means that the call for proposals will not go out this month, as had been hoped by commissioner Antonio Ruberti, but will instead be delayed until March or even June next year.

The EU also encourages eastern European countries to cooperate among themselves, and requires all proposals to have at least two central eastern European and one west European partners.

This requirement is often criticized in the east. Sklenička, who has a Copernicus grant, says that it is not the right way to help. He fears that the eastern partners may have little to contribute to these joint ventures, and so may learn very little from them.

Others, however, are beginning to see the benefit. A group of agricultural biologists from Poland, Slovakia, Hungary and the Czech Republic, first put in touch with each other through a Copernicus network, are now teaming up once again, this time without a western European partner. They hope to be able to persuade the EU to support a programme to develop plant breeding technology in central-eastern Europe, focusing on disease and stress-resistant crops.

One of the participants, Milan Bězdek, who is director of the Institute of Biophysics in Brno, sees this plant breeding programme as something that the east could do to help both its own agricultural development and agricultural problems faced by all countries of the world.

A.A.

Czech's streamlined academy

Prague & Brno. The Czechoslovak Academy of Sciences was founded in 1952 by the communist government, on the standard Soviet model. It comprised a series of research institutes, whose number had grown to 87 by 1989, and a learned society whose members supposedly included only the country's most talented scientists, but which in practice was deeply corrupted by politics.

Researchers at the academy did not have a bad life, although contact with the Western world was limited. The communists revered science which, they considered, would eventually solve all society's problems.

The academy was relatively free from ideology during the communist years. Indeed, it had been a sanctuary for scientists considered too politically incorrect to work in close contact with students at the universities. But, after the velvet revolution, the academy found itself less popular. It had to rethink its role quickly, and purge itself of the communist influence pervading its top echelons.

The first move came in 1989, when the academy declared itself an independent body, entirely free from state control. At the same time, it abandoned its discredited role as a learned society: it remains the only academy in central eastern Europe to have taken such a step. Most of the members of the general assembly were replaced and the new assembly voted in a new president, whose appointment was confirmed by the government. This independence and the new democratic structure were enshrined in a new act in 1992.

That has not been accomplished without pain. Among other things, the academy had to fight off a strong movement to dissolve it altogether and to merge its institutes with universities. But in retrospect, that was the easy part. Continuing reform proved more difficult.

Scientists became increasingly impatient of the failure of the academy's new presidium to deliver the required, and promised, structural reforms. Staff levels had fallen by not much more than a quarter, and independent evaluation of research at the institutes had not been systematically applied. Most of the staff that had left had simply found better jobs abroad or in the newly developing private sector. No institutes had been closed.

The academy was still distrusted by many as a privileged elite. It had no real friends in government and its increasingly obvious reluctance to get to grips with reform lost other support. Then the government slashed the 1993 budget by 15 per cent.

That announcement in the autumn of

1992 was accompanied by another shock: following a decree by the government that all state employees must prove they had had no connection with communist Czechoslovakia's secret police, Vladislav Hančil, one of the academy's first democratically elected vice-presidents, admitted that he had previously been an informer.



Rudolf Zahradník.

There was a crisis and the academy's general assembly demanded a new presidium. Jiří Velemínský, a plant physiologist, temporarily took the reins. By January 1993 he had seen through a fast, tough evaluation of the quality of research at the academy's institutes, carried out internally. Twenty per cent more staff were dismissed and 24 institutes were closed down completely.

Rudolf Zahradník the current president, is equally uncompromising in his attitude towards reform, instituting four-year cycles of entirely independent evaluations of each of the academy's 58 remaining institutes. The evaluation committees comprise half foreign scientists (mostly from Western Europe, to keep travel costs down) and half from Czech universities. The evaluation of 16 institutes began this autumn; the rest will be complete before the end of Zahradník's term of office in February 1997.

Pleased with his own success, Zahradník is critical of the many applied research institutes and universities in the Czech republic that have not yet institutionalized their own reforms. "The state gives applied research CKr3 billion every year, more than twice the academy budget, but there is no check on how effectively the money is spent. It makes my head reel", he says.

But morale is at last improving. Milan Bezděk, director of the Institute of Biophysics at Brno, says that he is optimistic about the reforms. In 1989, the academy

had 12,501 staff, 7,854 of whom were scientists. It now has just over 7,000, of whom 4,780 are scientists, 16 per cent of them with tenure. "The reductions look dramatic", he says, "but the quality of research is now much higher."

Zahradník is also happy, and says that the streamlined academy is functioning happily and efficiently. His one regret, however, is that the academy's budget is too low, which means that it can offer young scientists only very low salaries. That makes science unattractive to the brightest of the new generation, he says.

The privations seem fated to continue. Next year, there will be an eight per cent increase in salaries for all public employees, including academy scientists, but there will be no increase in the academy's general budget, which is being eaten away by an inflation rate of around 10 per cent.

Meanwhile, the government's promise, earlier in the year, of a gradual increase of spending on basic research by 20 per cent in real terms has been delayed, pending a more favourable economic climate.

Zahradník is 66 years old. He wants to spend what remains of his career finalizing the reform of the academy. At last month's fifth general assembly, he proposed the establishment of a new institute, for molecular sciences. "This is when you know a research society is working — when there are births as well as deaths", he says.

Alison Abbott

What's in a name?

Shortly after the velvet revolution, the very name 'Academy of Sciences' was anathema to Czech scientists. It recalled uncomfortable memories of when people were forced to work under the 'Soviet model': separation of research from teaching and control of research by the communist state. They were also conscious that the name engendered political distrust, and therefore lack of political support.

But times change. Earlier this year, Rudolf Zahradník, the current academy president, proposed that the academy's name should be changed to the National Science Centre. He thought it might appease the politicians, the public and the West. In any case, he also wanted the title of academy for the new learned society he is involved in creating, to bring it into line with Western learned societies.

Surprisingly, the idea attracted virtually no support. "Scientists feel that it has become our trademark", says Jiří Velemínský, head of the Institute for Experimental Botany. The stigma is no longer felt. Scientists abroad have also confirmed that the name is now happily associated with quality science in democratic central eastern Europe. □

Czech universities go slow

Prague & Brno. Czech universities have not enthusiastically embraced the opportunity to reform themselves along Western lines. On the contrary, apart from a few pockets of individuals, they have been steadfastly resistant to change. In the past four years, there have been fierce confrontations between universities and government.

Confrontation is now being defused, and democracy nudged along, with the help of electronic technology. Remarkably, the development of the new and much disputed university law, which is now going through parliament, was conducted by e-mail; anyone at any time could watch its progress and make their own comments.



Ondráček: backing the reforms.

The new law attempts to make good some of the mistakes made in the first days of democracy, which paradoxically served to block reform. Most important, it restores to university rectors their role as true heads of universities, allowing them, if they wish, to push through reforms without being overruled by deans and academic senates. It also introduces student fees, a development that has been hard for universities to swallow, but they will now probably be set at a fairly low level.

The new law firmly re-establishes the principle that universities should carry out research and sets up a new, government accreditation committee, whose responsibilities will include the reassessment of the status of the Czech Republic's 23 universities. The committee will recommend that those not carrying out research be regraded as technical colleges. The law formally introduces a PhD system and rules that degrees will be given by the universities rather than by the Academy.

Emanuel Ondráček, vice-minister for universities and research, thinks that the long battle to achieve some sort of compromise is over and that the bill will be passed by next spring, in time for the next academic year. He may, however, find his bill delayed by other governmental priorities.

It is not surprising that Czech universities are deeply conservative and protectionist. Czechoslovak universities bore the brunt of the communists' ideological assault on academic institutions. For 40 years — a whole generation — the universities were forced to abandon their traditions of free intellectual thought and instead had to kowtow to the marxist line.

Research was strongly discouraged and all but disappeared.

Small wonder that the reaction against this repression after the 1989 velvet revolution, known simply as "the November" by Czechs, was sometimes more emotional than rational.

In a rush of enthusiasm, the first democratically elected government of Czechoslovakia, as one of its first acts in 1990, passed a law giving universities complete autonomy. But the law also introduced an unworkable level of democracy into universities, by giving too much power to faculty deans and too little to rectors. At a time when tough decisions had to be made about staffing levels, about the competence of teaching staff and about general structural reforms, deans were too close to their faculty staff to be able to carry through decisions, while rectors, themselves not always totally reform-minded, right to tell them to be tougher.

Internal assessments of the suitability of individual staff members for their jobs were mostly formalities and nothing changed. There has been little turnover of staff in the past four years. In fact, staff numbers have risen from 11,216 in 1990 to 13,463 in 1993, because one new university was founded.

But university staffs are weary of change, weary of the stress of constant

assessment and competition — a lifestyle they are not used to. Recent elections for new university rectors reflect this depression. Overwhelmingly, rectors pledging to resist further reforms were elected.

Impatient of the universities' conservatism, the government tried in the summer of 1993 to force the issue by passing a "small amendment" to the 1990 universities act. This required all permanent staff members to be put on short-term contracts, to expire in September 1994, before which time each would be required to compete for his or her own job. Formal *concorsi* were held in the first six months of this year. In future, according to the new universities bill, only professors will have tenure.

To the frustration of the government and academy scientists, and to the glee of university staff who regarded the "small amendment" as an intolerable intrusion of the state on their autonomy, the move did not work.

Eduard Schmidt, rector of Masaryk University in the Moravian capital of Brno, 200 km south-east of Prague, describes the amendment as "a terrible thing — imagine, I was rector of the university, but I had no contract".

"But", he says, "we Czechs have experience in surviving. We had to survive the Austrian empire, Hitler, the communists...". In this spirit, most universities got round the amendment by exploiting the one-month delay between its enactment

Who should give PhDs?

In the Soviet model, imposed on Czechoslovakia in the early 1950s, the research degree was called Candidate of Science. It was similar to a PhD, but its criteria were less uniform and standards generally lower.

Ever since the velvet revolution, Czech academics have been keen to move to the Western PhD system for research degrees. In many places, PhD programmes have already started in anticipation of the provisions of the new bill on higher education now going through parliament. The Candidate of Science degree was finally laid to rest last year; scientists already part of the way through their programmes will be allowed to finish, but they may find that their certificates have little value.

But agreement that PhDs should be introduced has not been matched by consensus about who should award them. Before 1990, both universities and the Academy of Sciences trained students for their Candidate of Science degree, and both awarded them. In practice, most were awarded by the academy because almost all research was done there.

The ministry of education and science is now planning to take this privilege away

from the academy, on the grounds that universities should have sole charge of education. The academy, with its battery of relatively well-equipped research institutes and experienced research staff, will continue its deep involvement in practical training, but will no longer be responsible for the theoretical training.

Academy staff are profoundly disappointed that the decision about the awarding of PhDs, long awaited and still hotly disputed, has finally favoured the universities. Although the academy presidium is resigned to the situation, others continue to be furious. "It's like telling athletes that they can train newcomers, but they can't send them to the Olympics", complains Petr Boček, head of the academy's Institute of Analytical Chemistry in Brno, who also deeply resents what he feels to be the "violent and bad way" the academy's rights were taken away.

He speaks for many critics of the plan, who believe that the government wants to adopt a system appropriate in Western Europe, where universities always had a strong research history and had always awarded PhDs, onto the Czech academic system, ignoring the very different history in central Europe. □

and its coming into effect of 20 September 1993. They put all their permanent staff on short-term contracts immediately, so they would not have to face the *concorsi*. "The small amendment had virtually no effect on the number of employees at universities", says Miloš Chvojka from the ministry of education.

The Charles University went even further. In September this year, it ruled that candidates for professorships or assistant professorships must have taught undergraduates at Charles University for at least five or three years respectively, meaning in practice that posts at Charles can only be given to candidates already employed by the university.

This blatant protectionism, also viewed by some as xenophobia, has appalled a large part of the academic community: Schmidt himself does not approve. But Ondráček says that universities are now free to make whatever rules they want, for better or worse. "Whether it is rational is another question", he comments. His attitude is that the universities will have to learn from their mistakes.

The universities have been battling not just with the government but with the Academy of Sciences as well. After the velvet revolution, resentment against the privileges enjoyed by scientists in the academy research institutes came bubbling to the surface. And although every-

body agrees that universities without research are little more than extensions of high schools, it has been difficult to change the situation.

There are many reasons for this lag-gardness, some practical, some reflecting the old way of thinking. Few university staff have had experience of research; the few university laboratories were poorly equipped. At the same time, university staff had, and still have, immense teaching burdens that leave little time for them to think about research, let alone conduct it. And, despite their lack of experience in the field, university staff members have been reluctant to seek advice from academy scientists, with whom many felt at a competitive disadvantage.

According to Jiří Velemínský, director of the academy's Institute of Experimental Botany in Prague, irrational fears play a big part in the tensions between the academy and the universities. To put it plainly, which most are understandably nervous of doing in public, university teachers fear that they will be "shown up" by the expertise of academy scientists.

One manifestation of that is the failure of a plan to integrate academy scientists into universities that was introduced last year, when the academy was laying people off. The plan was met with hostility by universities, who felt that they were considered good enough only to be offered

second-rate staff. Although the scheme would have generously subsidized the employment of people from the academy, only a quarter of the CKr100 million allocated to the programme was taken up.

The result is that research has found its way into universities mostly on an *ad hoc* basis, thanks to the efforts of a few enlightened individuals and despite, rather than because of, the efforts of their universities' governing bodies (see accompanying article).

The government is now planning to intervene in favour of university-based research by changing the way in which cash is allocated. At the beginning of this year, Ondráček made 10 per cent of the budget contingent on research results, rather than purely on student numbers. This was hotly disputed at first, says Ondráček, but most rectors are now happy with the idea, and some would even like to see a rise in the proportion. Ondráček himself would be happy to see up to a third of the pay of university teachers dependent on their research performance.

Some universities have also taken budgetary steps to encourage research. Charles University in Prague, for example, has transferred 20 per cent of its overall budget into an internal grant system to which researchers can apply for funds.

A.A.

Institute living on its wits

Prague. The Prague Institute of Advanced Studies (PIAS) was founded officially in 1991 in a flurry of great optimism and idealism. It was to be an independent institute for applied research that would be closely associated with Czechoslovakia's first science park in Prague. It would have a staff of 350, including collaborators from universities with which it planned to form close links, and it would train up to 50 PhDs per year.

Four years down the road, its director, Peter Pechan, is still optimistic, but his idealism has been severely put to the test, and, after an encouraging start, things have not gone according to the original plans. For example, he has watched millions of dollars that could have helped set up the venture slip from his grasp because of government lassitude.

The idea for PIAS, and for a sister organization, the Slovak Applied Research Centre (SARC), arose after a meeting of Western universities and the Czechoslovakian Rectors Conference held shortly after the velvet revolution. A group of Czech scientists, disappointed at the vagueness of the meeting's conclusions, decided that something concrete should be done as soon as possible to help

Czechoslovakian science get onto its feet.

Pechan, who left the Czech Republic for Canada with his family in 1968 when he was 18, gave up his post as a principal investigator at the US National Institutes of Health to return to Prague to set up the institute. He would give the idea at least five years, he decided, knowing that such an ambitious programme could not be achieved overnight.

At first things went quite smoothly. The government and the Academy of Sciences both supported the establishment of PIAS as an independent associated organization. The government agreed to commission a report, funded by the European Union's Phare programme, on a science and technology strategy for Czechoslovakia, including a feasibility study on PIAS and its science park. The report, ready by the end of 1992, supported the investment of ECU10 million to the infrastructure for PIAS and ECU15 million for the science park, from Phare funds. It also made general recommendations on science policy, including the setting up of science and technology units within the government.

But by that time, Czechoslovakia was dividing into two republics; pressing political and economic issues pushed thoughts

of science to one side. The report was accepted by the government, but the minister responsible for research asked for a delay of six months in implementing it.

Six months have turned into nearly two years. With a simple letter of agreement from the government, PIAS could have received substantial EC funds; the research and industry directorates of the EU commission had agreed to support the report's recommendations. Lobbying has had little effect on a parliament distracted by other issues, and few in the scientific establishment have lent support.

Pechan saw that if he was to realize the dream, it would have to be without government help. PIAS now functions as a contract research organization with four of six planned research lines now established: ecological medicine, applied plant biology, immunology and peptide chemistry. There are 40 scientific staff. Evaluation of the effect of pollution on health in the mining area of northern Bohemia is the most advanced programme.

To take things further, PIAS has now formed a consortium with the hospital of the second medical faculty of Charles University and the local council in west Prague (where it is located) to develop a science park on a 50-hectare site adjacent to the hospital. The medical faculty has an option to become a partner. The consor-

tium will seek foreign partners to provide know-how and the financial guarantees. PIAS will eventually move into the science park if things go to plan. "We have proved we can do it without the active help of the government", says Pechan, but the whole thing could have progressed much faster and on a grander scale if the government had been behind them.

There is a small chance, however, that things may change: a few weeks ago, Maria Stiborova, vice-chairperson of the parliamentary left-wing block and a mem-

ber of the parliamentary foreign affairs committee, has tabled a formal question to government asking why the report, which cost ECU500,000 has been confined to a drawer. Pechan is not holding his breath for a change in government heart. Equally he is not going to give up. The five years he had mentally allocated to his project are running out, but he will probably stay a couple of years longer to see PIAS finally functioning inside a thriving science park. "I don't like to give up halfway," he says. **A.A.**

Clinical efficiency

Prague. Milan Elleder, who is a clinical biochemist, always wanted to combine research with his teaching activities at the Charles University medical school. He let nothing stand in his way, not even the dismal environment created by the communists antipathy towards research.

Since his appointment to the medical school's institute of pathology in 1964, Elleder has always kept a few research projects going, often initiating collaborations with his clinical colleagues. For 25 years he kept a low profile: "I hated the communist system and never tried to get into a position where I had to ask those guys for promotion", he says.

Unlike many in a similar situation, Elleder did not take sanctuary at the Academy of Sciences. He chose to stay at the university, because he enjoyed the contact with the clinic and with students.

He was not allowed to go abroad, but learnt English via correspondence with scientists in the West. This was tolerated, though the authorities "hoped the letters concerned only science".

Now Elleder has nearly everything he wanted. Last June he was made head of the newly established Centre for Inherited Metabolic Diseases, which combines the university hospital's diagnostic unit with his own expanding research laboratory. With West European help, he is moving away from enzymatic analysis of metabolic disorders towards molecular analysis.

The centre has a core of 12 scientists — six in diagnosis and six in research. Elleder is delighted to have attracted several young members of staff to the centre. Thirty-four-year-old Viktor Kožich, for example, spent two years at the University of Colorado, "a wonderful part of my life",

and thought he would never have the opportunity to do molecular biology again after his return to Prague in 1992. But he has found the chance at the new centre.

The centre's facilities have been built up with support from the Heidelberg-based European Molecular Biology Laboratory (EMBL). The tale is that one of EMBL's senior scientists, Wilhelm Ansorge, a graduate of Charles University awarded one of three chairs sponsored by the European Union, was encouraged to spend as much time as needed to establish a high-standard laboratory in Prague.

The European chairs, administered by the Institute for Human Sciences in Vienna, provide salary for one year and generous equipment grants (ECU150,000) to a scientist in Western Europe to promote high quality research at a university in central eastern Europe.

Elleder is enormously grateful. "It is very important to have someone we can turn to", he says. Ansorge travels frequently to Prague, often bringing young scientists from EMBL with him to contribute practically. But he is not complacent: "I would like to be optimistic, but I realize there is still a lot before us", he says. The biggest problem is one of attitude; people grew accustomed to how things were under communism and are afraid of change."

Elleder still prefers to keep a relatively low profile, conscious of the time-consuming and frustrating nature of university politics. His only concession is his acceptance of the position of vice-dean for the new PhD programme at Charles University.

The Czech Republic is about to abandon the old Candidate of Sciences degree which, says Elleder, was more of a professional qualification than a true research degree. He wants subject areas of PhDs to centre on pathogenesis and therefore advance basic knowledge, and to exclude routine subjects such as surgery.

His remaining ambition is to establish a graduate school in the medical faculty. He has support from the university rector's office, and from Emanuel Ondráček, vice-minister for universities and research, but the plan must be voted through the more conservative academic senate, which is not enthusiastic, fearing that it would allow too great a concentration of power.

Elleder will spend as much time as necessary making sure the graduate school becomes a reality and that standards of PhD work remain high. He is conscious of the opportunities and freedom that now exist and which, he believes, should not be allowed to slip away. When he applied to go to university at 17, the authorities noted that he was from a bourgeois family and sent him to work in a factory for a year "to get to know workers". His own son at the same age was able to go to school in the United States on a year's exchange. **A.A.**

Why be a scientist?

A RECENT opinion poll in the Czech Republic revealed that science is regarded as the fourth most admired profession. This is a surprise, and perhaps an indication that the star may once again be rising for researchers.

Science was a fashionable career under communism. Indeed, science was regarded — and loudly hailed — as a major pillar on which communist societies are founded. But with the collapse of the old regime, scientists began to be regarded with suspicion. Worse, pseudoscience began to flourish, and continues to flourish: there is a suspicion that, having been banned under communism, it must be good.

To make things worse, the state offers scientists miserable wages. There is virtually no unemployment in Prague, and much more can be earned in the private sector. So it is not hard to imagine the difficulties scientists face in trying to persuade bright young students to turn to a career in research.

Scientists at the academy are allowed to spend up to 50 per cent of their time working elsewhere to supplement their

income, but that is not a satisfactory solution. Instead, to regenerate their ageing stock, the only hope for research institutes is to fire their young students with enthusiasm.

One way is to use the many opportunities in the Czech Republic to send young students abroad to study. Around half of the republic's postgraduate students have travelled abroad under the European Union Tempus scheme. They come back liberated. "You can always tell which students have been abroad", says Jiří Veleminský, head of the Institute of Experimental Botany in Prague, "because they answer you back, and criticize you."

By contrast, the usual Czech student has been taught to be passive. Martina Rothova, a research student at Charles University's department of botany, has made several trips to Munich to learn molecular biology techniques. She has been amazed at the helpful attitude and enthusiasm for science she has found at the Max Planck Institute for Biochemistry. "You don't get this at home", she says. □

Romania after the Ceaușescu

Bucharest. The fall of Nicolae Ceaușescu's repressive regime in December 1989 was the last in the series of domino-like events that has transformed the political and social climate of central Europe. That Romania should have been the last in line was natural. And in the event, Romania was the only central European country in which the the revolution was violent.

Romania's recent history is unique, and far unhappier than that of its neighbours. Alone in the eastern bloc, Romania (which has no borders with the West) had gone its own political way in the two decades before the revolution. Indeed, Ceaușescu fell out of favour with Moscow when he refused to let Russian troops pass through Romania for the invasion of Czechoslovakia in 1968. But if less under the Soviet thumb, Romania suffered much more than any other country from its home-grown dictatorship, which systematically set about destroying its own culture.

Science was a conspicuous victim. It is difficult now to grasp how the bizarre events of the Ceaușescu years could have happened and how a country so culturally rich — Bucharest used to call itself "the Paris of the east" — could be brought down so far. Ceaușescu seems to have had an irrational fear of science, presumably because it required intellectual thought in an area not obviously open to political control. His wife, Elena, who liked to call herself a top chemist but had no genuine qualifications, personally controlled all Romanian research.

But independence from Moscow brought one important benefit: technology development contracts with the West. Thus a Candu nuclear power station was built with assistance from and collaboration with Canada. Romania also had a joint project with British Aerospace to build the BAC 1-11 passenger jet; Rolls Royce engines were manufactured near Bucharest. The reasonable purpose of these contracts was to transfer technological know-how from the west.

On the other hand, the goals of science and research in Romania were grossly distorted. All efforts were directed towards applied research, little to basic research and none to social science. University research was negligible and politics corrupted all the levels of the scientific enterprise.

Even the institutes of the Romanian Academy, responsible for the minimal basic research effort, were affected. In most other countries, the academies retained at least some measure of intellectual autonomy, but not in Ceaușescu's Romania. Indeed, the academy, founded in 1866, became a Soviet-style state-con-

trolled institution in 1948. Its name was changed to the Academy of the Romanian People's Republic and about 100 academicians were promptly expelled.

Thereafter, politics determined the membership of the academy. The last member to be elected before the revolution, in 1974, was Elena Ceaușescu. She immediately forbade further elections and set about dismantling the academy, transferring its institutes to government ministries and putting them under the direct control of the National Council for Science and Technology, a political body set up in 1966 of which Elena Ceaușescu became head in the 1980s. That gave her tight control of the basic research institutes and of the hiring and promotion of scientists.

While Elena Ceaușescu was restructuring the academy institutes, her husband was reorganizing industrial research. He separated research and development



Doru Palade, must "pick up the pieces".

units from industry and set up instead 'industrial centres' within which one or two research units were put at the disposal of a large number of industrial units, which were not allowed to have their own research departments.

"Ceaușescu did something that other communist countries never achieved", says Doru Palade, minister for research and technology, "he managed to isolate industrial research."

After the revolution, science was completely reconstructed, but hardly in an ideal way. The new government's first knee-jerk response was to abolish the National Council for Science and Technology, but that was not replaced by a new structure for two years. During that time, says Palade, reform stood still.

Even now, the reform process is painfully slow. But the government is under external pressure to speed things up. It has agreed to implement recommendations by the World Bank following its study of science and technology in Romania. These include the establishment of effective and transparent mechanisms of funding research, a competitive grants system and proper evaluation of research.

But people are conservative. Many hold that Romania is being held to ransom by foreigners wanting inappropriately to impose a Western system on a country with its own distinctive history. "You can't simply transpose Western models", says

Virgiliu Constantinescu, president of the Romanian Academy, "It would be like trying to translate poetry."

One of the first laws passed after the revolution (in the first month) returned autonomy to the academy. But to the chagrin of many, particularly the World Bank, the academy used its autonomy to recreate a structure similar to that of 1948. It remains a learned society, but has reinstated members expelled after 1948 and has expelled those who, after the revolution, were convicted of working for the secret police. But there is no mechanism for assessing the worthiness of others elected as academicians during the communist regime.

The academy has also reclaimed the research institutes that had been under its umbrella between 1948 and 1974. Moreover, it expanded its portfolio to take in other institutes attached to ministries, without proper evaluation of their work and aims. It now finds itself less and less able to support this large network, which now totals 72 institutes and research units.

There has been no evaluation of research institutes, as there has been in other central eastern European countries. Nicolae Simonescu, director of the Institute for Cellular Biology and Pathology and in charge of the reform programme in the academy, says that attitudes are changing a little, but only slowly. Constantinescu agrees that there is a psychological problem: people are not yet convinced of the importance of evaluation, he says.

The academy is now so short of funds that many scientists say that their day-to-day lives were easier before the revolution. This is ironic, because the academy is the only one in central eastern Europe that is potentially very wealthy. Before 1948, it owned large amounts of property in Bucharest, elsewhere in Romania and abroad. Constantinescu is seeking to get these investments back, so far with little success.

But the recreation of the academy has at least meant that more of the budget is now spent on basic research; the proportion rose from 10 per cent in 1990 to more than 20 per cent in the succeeding years. Worryingly, it is now declining again.

Applied research has also escaped reform. After the revolution, applied research institutes were increasingly short of funds and were exhorted to find new research directions more appropriate to the times. But not much has changed. The applied research institutes still absorb a large part of the science budget, but have not yet been exposed to the rigours of formal evaluation.

Staff numbers have declined, but not as dramatically as elsewhere in central eastern Europe. At the Institute for Atomic Physics, for example, staff turnover since 1990 has been barely 20 per cent. The

staff roster, now 3,500, has declined by less than that. The director, Alexander Glodeanu, asks, "How can anyone ask me to fire a physicist who will not know how to set up a company or to work at anything less specialized?"

Even so, science at the institute is looking up. Its microproduction centre, founded to manufacture one-off pieces of equipment otherwise available only in the West, has been closed down. Instead, the institute has taken on projects newly recognized to be important, such as the environmental impact of tritium contamination from nuclear power stations. It has also developed a range of collaborations with scientists in the West, often through European Union projects. But, despite the evident inefficiency, the Institute of Atomic Physics is a shining example to the other applied science institutes.

That is one reason why Palade, the research minister, is trying to orchestrate the return of some industrial research units to industry. But, given the three-year delay in starting on this task, it is now not as simple as it might have been, he says. Some of the industry centres' research units have set themselves up as independent contract research companies. Some have disappeared. However, there have been some successes this year.

For example, Avera, a very large company manufacturing pumping equipment, has taken back a research and development unit, as has the major hydroelectric power company Resita. "We expect these trends to continue", says Palade, "but the problem is that the mother company needs to be very strong, while our industry is generally weak."

As reforms drag slowly on, scientists are getting used to the freedom of post-Ceausescu life. The dictator's fear of science and technology had bizarre consequences, now frequently recalled for amusement. How can you explain without despair, for example, that the president of a large country, with a strong cultural tradition, so hated computers that it was forbidden even to use the word on television? Or that the word 'fossil' was similarly banned, in case audiences were tempted to make derogatory references about the ageing Ceausescu?

The situation sounds ludicrously comical now, but scientists remember yet more vividly the atmosphere of fear in which they had to work. "We always had to be careful how we spoke, because you never knew who was an informer", says Valeriu Zoran, a research director at the Institute of Physics in Bucharest. Scientists no longer have to keep looking over their shoulders.

Another benefit is the freedom to travel abroad. But that remains an abstract freedom. Few can find the funds to travel.

Alison Abbott

Uncontrolled university boom

Bucharest. Universities in Romania suffered the usual fate in communist countries: they lost their independence and they lost their research base.

After 1948, the universities were purged and many staff were sent to jail. Many others left the country. Many courses, particularly in the humanities, were stopped and subjects such as journalism transferred to communist party schools. Research institutes attached to universities were transferred to the Romanian Academy.

According to its rector, Emil Constantinescu, the University of Bucharest suffered more than any because it was Romania's prime cultural symbol. It was founded 130 years ago, on the site of the College of Royal Studies, which was itself established in 1694.

Now a member of parliament as well as being president of the Romanian Rectors Conference, Constantinescu has been the main force behind the post-communist reform of the university system.

There was no witchhunt of university staff after the revolution, he says, but nonetheless many lost their jobs. The students played the biggest role in choosing who stayed. Parallel courses were set up, and some professors whose courses did not attract enough students were asked to leave. Others were offered the chance to improve.

But those who left did not find themselves without a job. There was a very curious phenomenon in the years following the revolution. The increased demand for student places meant that student numbers in all state higher education institutes rose from 7,000 in 1990 to 30,000 in 1992, and that was still not enough.

Further demand was met by the establishment of private universities which started to spring up everywhere. It is estimated that there are over 60 such institutes, enrolling possibly 100,000 students.

Using makeshift premises — a cinema during the day or a secondary school during the evening, for example — these private universities are staffed partly by former state university professors and partly by academics with full-time jobs, either at state universities or institutes of the Romanian Academy, who need to top up their meagre incomes.

The flourishing of these private univer-

sities is almost an accident. It has been made possible by a law passed in 1990 that legalized private higher education institutes. The government had hoped the result would be private tutoring for university entrance, forbidden in Ceausescu's day. The outcome was not predicted, and is potentially disastrous for Romanian education standards.

Though not accredited, the private universities claim to issue degrees. Many offer courses very similar to those offered in state universities, others offer unconventional subjects such as homeopathy. Most are also extremely ambitious, offering programmes in popular and expensive subjects such as medicine or architecture. Inevitably, the quality of the courses is very questionable. "They are a real problem," says Constantinescu, "because the consumer interest is not protected." But things may soon change; Romania's new accreditation committee is busily assessing the suitability of all institutes to offer recognized degrees. A new law also allows students at private universities to sit their final examinations at a state university if they prefer.

Within the state system, the universities have since 1990 been busy reconstructing their curricula, introducing courses in the humanities, Western languages and business studies. They have also been striving to reintroduce research. Twenty-five research centres have been set up in association with departments at the University of Bucharest. Links with research institutes of the Romanian academy are being forged, and PhD programmes being introduced.

These changes are being conducted with a dramatically shrinking budget. The 1992-93 budget of US\$92 million was 30 per cent lower than in the previous year and the downward trend continues.

A further strain on the system arises because a large proportion of teaching staff were promoted after the revolution. That is meant as compensation for yet another bizarre peculiarity of the Ceausescu years. As one means of suppressing the academic community, Ceausescu saw to it that there were no promotions for academics after 1974.

Inevitably, there was a clamour for restitution after 1990. A generation of academics held at the lowest pay-scales for most of their careers wanted promotion immediately. Viriligiu Constantinescu, president of the Romanian Academy, who was rector of the Polytechnic of Bucharest from 1990 to 1992, says that he tried unsuccessfully to ask for patience, warning academics that promotions should depend on a proper assessment of performance. But there proved no way of controlling the demand.

A.A.



Constantinescu — cautious reformer.

Bootstrapping a new institute

Bucharest. The jewel in the crown of the Romanian Academy of Sciences is the Institute of Cellular Biology and Pathology, run by the husband-and-wife team Nicolae and Maya Simonescu.

A thriving centre, with 130 staff, 50 of them scientists who have mostly worked abroad, the institute is one of the very few in Romania performing world-class research. It concentrates its efforts on the molecular and cell biology of the cardiovascular system, with particular reference to atherosclerosis.

The centre did not attain this position easily. Its present status is entirely the product of the determination of its director to provide a haven for good science in his home country despite the almost impossible political situation.

Nicolae Simonescu made his escape from Romania in 1970 when, after a year-long struggle, he got a visa to visit the United States. He spent the next three years as a research fellow at the Rockefeller University and then moved to Yale, where he eventually became a professor.

Despite his success across the Atlantic, Simonescu always wanted to return home to help make it possible to do good research work in Romania. In 1972, he sent the government a proposal for the building of an institute of cell biology, in which there were virtually no studies anywhere in Romania. His association with

George Palade, a Nobel prizewinner and Romanian expatriate, helped his cause; the following year, the government approved the proposal in principle.

Simonescu agreed to return to run the institute under certain very strict conditions, the most important being that he would be allowed to spend a part of each year in the United States, and that he would be able to send his young scientific staff there for training.

After numerous delays, during which decisions were repeatedly made and unmade, the institute, reasonably equipped, finally came into being in 1979. The Simonescus came home. But they were immediately told by the Ministry for Health and Education that funds had run out. The institute would receive funds only for salaries and maintenance costs.

For the succeeding decade, the Simonescus survived on substantial grants from the United States — for a time, they were the only institute in eastern Europe to hold grants from the US National Institutes of Health — and brought vast shipments of equipment and journals back with them from their annual visits to the United States. Customs records show that, between 1979 and 1992, they transported 14,000 kg of material, worth US\$33,000.

In addition, Simonescu managed to send 16 young scientists to work in laboratories in the United States during the

1980s. Surprisingly, none defected: all returned to form the core staff of the institute.

The Simonescus established a number of traditions in their institute during the 1980s, in defiance of the government. One was to require all scientists to give regular progress reports of their projects in English, to ensure that language did not become a scientific barrier. They also held regular US-Romanian scientific workshops, bringing in US scientists, using US money.

The Simonescus have sought to nurture a broader appreciation of culture. They play taped classical music concerts in the common-room with information sheets about the composer taped to the tables, and have used their auditorium to hold cultural, as well as scientific, events. They celebrate Christmas — complete with Christmas tree and Santa Claus — for the staff and their children and insist that everyone takes the day off.

These actions were regarded by the old regime as provocative, but they were tolerated. But as time went on, the institute began to be regarded with more and more suspicion by Ceausescu, culminating in his declaration in September 1989, at the height of his paranoia, that Simonescu was a US spy, selling Romania's scientific secrets. His visa, needed to allow him to complete his autumn lectures at Yale, where he was still a visiting professor, was withdrawn. After pressure from US scientists and the US embassy, he was belatedly allowed to go. "It was a very scary time", he remembers, thinking it would be his last trip. But when he returned in January 1989, the revolution had changed everything.

There can be no comparison between conditions now and then, says Simonescu, even though the present is very far from ideal. Funds are now decreasing, not increasing. And although there is now complete freedom to travel, as often as not the young scientists who continue to be sent abroad for training no longer return. Indeed, some of those who resisted the temptation to defect during their periods abroad in the 1980s now want to leave. "They do not complain that they cannot do good research here", says Simonescu; "It is mostly because the salaries are too low."

The Simonescus remain undaunted. Their strategy is to keep training new people, even if the returns are now likely to be lower.

Nicolae Simonescu is also devoting energy to encouraging reforms within the academy as a whole. He is also in charge of Romania's biotechnology programme and has arranged for a small amount of its funds to be set aside for competitive grants. This is, for the time being, the only example of competitive project money in Romania.

A.A

Taxing times for research funds

For the first two years after the revolution, there was no government structure with specific responsibility for science. Elena Ceausescu's hated National Council for Science and Technology had been abolished. Science was in a vacuum.

With the election of the second post-revolutionary government in 1992, a Ministry of Research and Technology was established. That, in turn, is now trying to develop a science policy. In time-honoured fashion, the ministry has created two advisory councils, one for applied and one for basic research, each of which includes a network of specialist committees drawn from the scientific community. The plan is to set research priorities and supervise research programmes.

The committees have not yet reached agreement on a general policy, but ideas include the establishment of a Romanian Fund for Science to provide Romania's first formal competitive project money, and the establishment of an interministerial Council for Science and Technology, to define research and development priorities and allocate funds accordingly.

Meanwhile and inevitably, the budget

for science in Romania has fallen dramatically. In 1990, it was at its peak of 2.6 per cent of GNP, but by 1992 it had fallen to 1.7 per cent and is now around 0.6 per cent of GNP. The leader of one of the main parties in the governing coalition recently pledged support for an increase to 1 per cent, but it is understandable that the goal is, at present, unrealistic.

It is less understandable that the diminishing funds have been so disastrously administered. While the Romanian Academy has always had its own direct budget, more than half of the rest of Romania's research has been topped up by a 1 per cent tax on private and state companies, imposed in 1991. The tax was modelled on Ceausescu's New Technology fund. In the event, in post-revolutionary Romania, the tax has proved impossible to collect. The result is that the research ministry has had to take out loans, which it can ill-afford, to pay its research institutes.

Mercifully, the government has agreed to comply with a strong World Bank recommendation that the tax be replaced next year by normal budget support. □

Old academies of sciences in transition

The academies of sciences in the countries of the former Soviet Union, Eastern and Central Europe no longer have a decorative role in national affairs, but are remarkably confident that the worst is now over.

Sofia. The 125th anniversary of the Bulgarian Academy of Sciences (BAS) was celebrated on 12 October by a gathering of dignitaries from other academies in the newly democratic states, and was thus an opportunity to judge how the rest of Central Europe is adjusting to the collapse of communism. If happiness is a carefree state of mind, it was not much in evidence at the anniversary celebrations.

For Bulgaria, President Zhelio Zhelev asked an ironical question. The Bulgarian Literary Society, the precursor of the BAS, was founded a decade before the state itself, in the unfavourable atmosphere of Ottoman domination by the devotion of Bulgarian emigrés scattered all over Europe. So why, he asked, is Bulgaria now, while governed by its own democratically elected parliament, unable to support its existing scientific institutions?

Part of the answer was given by Dr Alexander Jordanov, the speaker of the parliament: "the real power now belongs to the people who have not read two books in their lives", and who are afraid of "the internal light emanating from the scientific community to illuminate society at a time when the dark side of the Bulgarian soul has come to the surface of public life".

The president of the Romanian academy, Virgiliu Nicolae Constantinescu, claimed for his country some of the credit for the origins of the BAS, saying that Romania's liberal climate in the middle of the nineteenth century allowed Bulgarian intellectuals to gather in the town of Braila to draft BAS's statute (a synthesis of preliminary work at Odessa, Vienna and Bucharest) only three years after the

Romanian Academy of Sciences was created. That institution, active in achieving the unity of the Romanian language among other things, became a pillar of Romanian nationality and the state.

But then, after the Second World War, came "a disastrous setback". In 1948, the existing academy was dismantled and a new one installed, with directly nominated members. "Over 100 former members were excluded", said Constantinescu, and were replaced by "leading communist figures, of little or no achievement" who made the academy into an ideological body. Most of the former members were persecuted, "and some ended their lives in jail".

But Constantinescu said that the true demolition of the Romanian Academy of Sciences began in the late 1960s, when all the research institutes were virtually closed down. By the 1980s, the total personnel had been reduced to seven, including the president and the night-watchman. The result was a massive emigration; Romanian mathematicians today hold professorships at 300 universities in 39 countries. By December 1989, the 90 surviving members had an average age of nearly 80.

An engineer by background, Constantinescu believes that the situation is now on the mend. With so few commitments to the past, the academy has arisen from nothing since the 1989 revolution. Some 130 corresponding and full members and 31 honorary members have been elected. Constantinescu thinks it fortunate that there were so many people "unspoiled by the former communist regime" and boasts that neither he nor his four vice-presi-

dents and secretary-general belonged to the old academy.

Apart from the traditional recognition of excellence in scholarship, the new academy is meant to be a repository of valuable historical and cultural material and to give competent answers to major questions of national interest. (In that role, it is responsible for a dictionary of the Romanian language, and is proud that it is now working on the letter 'Z'; work on 'A', 'B' and 'C' had been completed in 1928.)

Constantinescu's academy will also support research in natural sciences and intends to be active internationally, being a founding member of ALLEA (Alliance of European Academies), under whose patronage it plans to organize an international workshop, "Academies in Transition", in April 1995.

At the other end of Europe, the Estonian academy seems at the other end of the spectrum of contentment. Its president, Arno Koorna, told his Sofia audience of the problems caused by the ambition of the ultra-nationalists to "break down all the institutions of communist era". They wish to turn the present academy into "a sort of club", depriving it of its research institutes and therefore of state finance (now 0.4 per cent of GNP, which allows it just to survive).

Koorna believes that the Estonian parliament's antipathy towards science is explained by the youth of the right-wingers, who are mostly under 30. They do not appreciate, he says, that Estonia's only hope of becoming a wealthy state is to spend its last penny on education, sending talented young people to the best universities and using their knowledge for Estonia's benefit. But the parliament has elected as chairman of the Committee for Science a man called Sulev Alajoe who was expelled from the Technical University for poor academic attainment.

The Estonian academy's budget for this year of 50 million kroons is approximately US\$4 million, less than the \$5 million given to Estonian scientists by George Soros through his Open Society foundation. The average academy salary is now only 1,700 kroons (13 kr = \$1) a month, with the result that there is a 'brain-drain' to other countries and other occupations. In the past three years, the academy's institutes have lost about 30 per cent of their staff, although the total number of employees in scientific organizations is still approximately 10,000, half of them directly involved in research activities.

Koorna accepts that Estonia cannot



The home of the Bulgarian Academy of Sciences.

finance all the fields in which it was active "when it was a part of an empire", but he is disappointed that, despite an evaluation by the Royal Swedish Academy that concluded that "almost 80 per cent of all the Estonian academy's projects are of a reasonable standard, we are now to restrict their number". He particularly regrets that the Institute of Marine Research is now compelled to sell its well-equipped research fleet.

The optimism of Romania and the bitterness of Estonia were echoed by other academy representatives from the former socialist bloc at Sofia. The antipathy towards science of the newly elected democratic parliaments seems commonplace, as does the lack of finance and the resulting brain-drain that accompanies it. The difficulty of research that requires expensive equipment and the breaking of scientific links with former partners, mainly Russia, were other common complaints.

The painful withering of familiar links with Russia is caused not by politics, as one might think, but by the lack of money. East European states can no longer afford to promote scientific contacts by covering travel expenses or even accommodation costs for researchers invited for a conference or a workshop, by contrast with governments and other organizations in the West. Is the 'golden curtain' likely to prove even less transparent than the 'iron curtain' it has replaced?

But there are some advantages in the new situation. The centralized control of science is less onerous and, especially in the humanities, ideological dictate has gone. The number of scientists may have diminished, but many of those who have left were not scientists at all. Now, when most academies (except the Russian and Bulgarian) do not pay large pensions to their members, there is no reason to elbow one's way into them unless one has a real interest in science. Indeed, many academy representatives agreed with Koorna's declaration that "the present number of scientists in Estonia is optimal".

Indeed, the productivity of many academies' researchers, measured by the number of publications in journals, has increased in the past 3–5 years. "I can safely say that the worst is over for science", said Jordan Malinovski, president of the Bulgarian Academy of Sciences and the host of the Sofia meeting.

But that may have been too optimistic. There was a reminder of the previous era in the appearance of an incongruous pair: a short old man with a hearing aid invariably accompanied by a tall and much younger man. The former, Dr Demirai, the president of the Albanian Academy of Sciences, was silent. The latter, who appeared to have no name, constantly repeated, "The president is busy, no interviews available". □

Bulgaria — cutting to the bone

Dr Jordan Malinovski (inset), president of the Bulgarian Academy of Sciences (BAS), considers Bulgarian science to be in a state of collapse. Openly, he blames this on the totalitarian regime that oriented industry and agriculture to suit the demands of the Soviet Union.

Now that the colossus has fallen, it seems that nobody wants Bulgarian goods because of their poor quality. Production has thus decreased dramatically. Moreover, scientists, who were once paid by the state for their contribution to the national economy, have lost government support.

There is worse. Since 1912, the BAS has been granted exemption from taxes and import duties on all essentials from abroad. Last April, the new non-totalitarian government proposed a law to the now democratic parliament depriving Bulgarian science of its privileges. It was passed.

The result, Malinovski says, is that \$500,000 worth of scientific equipment "is now languishing in the customs' possession". Although the equipment has been given for nothing by foreign colleagues, BAS cannot afford the huge taxes and duties required, and so cannot collect it. That is the injury. The insult is that BAS is "even compelled to cover the expenses of keeping it in the customs warehouses".

But the real concern of Bulgaria's "first scientists" is their budget support. For 1994, the government allotted (but has not yet paid) to BAS only 1 billion leva (66 leva = US\$1). A further 90 million

leva comes from the National Scientific Foundation (NSF). And of the total, 75 per cent will be spent on salaries and 18 per cent on electricity, water and other essential services.

In other words, there are no funds for anything but mere survival, and certainly little for the purchase of scientific equipment. Yet strange as it may seem, nobody is likely to try to alter the April law.

In the present political situation, in which two major rivals, the Bulgarian Socialist Party (BSP) and the Union of Democratic Forces (UDF), are practically equal in power, there is little faith in the wisdom of parliament.

"Don't bother with Philip Dimitrov and Philip Bokov. Vote for Philip Morris!". This is the motto of a popular television show, Ku-Ku, directed by Luben Dilov Junior, son of the chairman of the Bulgarian Writers Union. (The first two Philips are respectively the chairman of the UDF and one of the leaders of the BSP).

So the BAS is left with only one option: cutting costs. Eighteen institutes have already been closed down, and others await their turn. But this gives rise to another problem, unemployment, which is increasingly hurting the Bulgarian scientific community.

Yet ironically, the academy has banned institutes from earning money by creating companies to manufacture goods based on their investigations. The reasoning is that it could lead to unlawful enrichment of the scientists involved. **Carl Levittin**



How to survive in business

Dr Rumen Tsanev is one of Bulgaria's scientists who have joined the commercial sector. After 30 years, he left his post as a director of the Molecular Biology Institute to join Pharmagen, a company founded by the institute, but now independent of it.

Bulgarian businessmen have invested US\$1 million in Pharmagen to finance the manufacture of γ -interferon — effective against many viral diseases, in particular herpes — by a genetic engineering technique developed at the institute. Because labour is cheap and the technology effective, the medicine is a tenth the price of its equivalents. This enables it to be used against leishmaniasis in India, Africa and Latin America.

Tsanev claims that γ -interferon also represses collagen synthesis and that it is already used in clinics to cure certain diseases that cause the skin to overproduce collagen. It has also been tested against immunodeficiency in one Bulgarian military hospital, and has proved to be effective. If

there were more HIV-infected patients in Bulgaria, γ -interferon would no doubt be being tested for its anti-AIDS properties.

According to Tsanev, Bulgaria can still make scientific discoveries because, under the totalitarian regime, they could buy expensive equipment, build large reserves of reagents, travel frequently and contact colleagues. "We use all that now"; that is Tsanev's secret of survival.

The institute receives 11 million leva from BAS and 1 million from NSF, and spends 6.5 million on salaries and 2.5 million on rent, electricity, water and other essentials. What is left (around US\$20,000) is almost nothing.

"We were lucky in that UNESCO presented us with scientific equipment worth US\$100,000 before April", says the former director, "But now we have to ask our colleagues to refrain from such gifts, thanks to our present government, which seems to have forgotten the word 'science'." □

Ecology benefits from decay

Sofia. Historians of ancient Thrace (whose research institute the BAS has closed) will tell you that the Bulgarians' ancestors were carefree, almost lazy; they sang, danced, and had plenty of holidays. So, the story goes, they were the last to ask for their land when God was distributing it. The Almighty by then had none left, and was forced to give them some he had reserved for himself.

That is the only explanation why Bulgaria possesses everything: mountains and valleys, sea and rivers, forests and fields covered by blossoming roses; why it occupies the crossroads between north and south, east and west.

True or not, that gives science a target: to use all means, theoretical and experimental, to save this God-chosen land. That, at least, is how Dr Yordan Uzunov sees his role. As a senior researcher at the Institute of Ecology of the BAS and Deputy Minister of the Environment, he uses both scientific and administrative approaches.

Uzunov even believes that the present situation provides a unique opportunity. "Today's dormant industry and agriculture pollute neither water nor air. . . the ecological situation is now better than anyone can remember." He says the task will be to maintain that state of affairs when the economy revives. Unfortunately, the percentage of GNP allocated to ecology is also falling: in 1992 it was 0.45 per cent, in 1993 0.4 per cent, and this year only 0.29 per cent.

And there are not roses everywhere. As a remote province of the old Eastern bloc, Bulgaria had to carry out ecologically hazardous production for the rest of the empire. One example is uranium mining. Economically, it was absurd. The cost across the globe of producing a kilogram of concentrated non-enriched uranium is between US\$17 and US\$26, but in Bulgaria it is US\$250. It would have been much cheaper to mine uranium in Armenia.

But political reasoning, and the wish to move 'dirty' production to the periphery, won. Now Bulgaria will require US\$160 million and 10–15 years to recultivate land currently enclosed by barbed wire.

The other ecological problem is energy production. Bulgaria has hardly any naturally occurring oil or gas, and its coal is of poor quality. Of the country's electricity, 46 per cent comes from a nuclear power station in Kozlodui. But 47 per cent of the population is afraid of radioactivity and 70 per cent think the government is deceiving them by using a second nuclear power station, built secretly and well-hidden.

Opinion has been powerfully influenced by people such as the French oceanographer Yves Cousteau, who visited Bulgaria to protest against the opera-

tion of Kozlodui. He is believed to have ceased campaigning after the Kozlodui station bought some French equipment. But as things are now, proper station maintenance is impossible.

The National Ecological Foundation, founded in March 1993, is not helping matters. It is supposed to collect money from those polluting the environment, but everyone manages to postpone paying fines. The foundation's revenues instead come largely from duties levied on the owners of older cars.

As always, Bulgaria's position at the crossroads of Europe brings Bulgaria both trouble and income — the flow of such cars through the country is considerable. Last year, NEF collected 108 million leva in this way, returning 67 million leva to local government organizations for the improvement of the ecological situation.

But that is a drop in the ocean. Bulgaria hopes to take the route already followed by 16 other countries of trading foreign debt for domestic spending on ecological projects. Poland is the envied example in this part of the world. A fraction of Bulgaria's US\$14-billion foreign debt thus converted could do a great deal for environmental protection. But Bulgaria differs from Poland in that its main creditors are not other governments but private banks and companies not overly concerned with ecology. **C.L.**

World Cup — an excuse to celebrate

Foreign countries have always played an important role in Bulgarian science. The Bulgarian Academy of Sciences was itself founded abroad — as recounted by the president of the Romanian Academy (page 608).

After the liberation of Bulgaria in 1911, that became the Academy of Sciences, which celebrated its 125th anniversary on 12 October this year, a few weeks before the 125th anniversary of *Nature*. The president of Bulgaria, Zheliu Zhelev, and the director general of UNESCO, Dr Federico Mayor, were both present.

Many Bulgarians considered the celebrations to be an unaffordable luxury, but accepted that the presumed wishes of the nation's ancestors should be taken into account. The winning by the Bulgarian team of the bronze medal at the World Cup football championship in July led to a nationwide festival. Not everyone here is a soccer lover, but most are tired and desperately need a pretext for celebration. Why should not the events of this October give Bulgarian science a new impetus? □

A phoenix flies

Sofia. Enterprise may be an unfamiliar plant in Bulgaria, but it can sometimes flourish marvellously.

Perhaps the best example is *Orbita*, a 16-page weekly founded in 1969 and for decades the only paper in Eastern and Central Europe completely dedicated to science. It was one of the country's four most popular publications, with a peak circulation of more than 160,000 in 1990.

But in the autumn of 1991, it was closed by its publishing house, owned by the recently defunct Young Communist League. The very next day, the editorial staff produced that week's edition, newly registered in strict accordance with the law, which gave their subsidiary private firm Omega the right to publish a weekly now called *Nova Orbita*.

The money they had managed to save allowed them to continue for a month or two until they were rescued by two truly wealthy firms, Diamex and Dizal, and two private individuals, Petko Todorov and Slavcho Ivanov. Now a new enterprise called Diomega produces the newspaper.

But still there is not enough money, with the result that *Nova Orbita* is subsidized by publishing a magazine containing crosswords, puzzles and the like, and by selling advertising space. The staff consists of only 6 people compared with the former 35. Everybody has to work hard.

Yet there are some benefits in this arrangement. Nikolai Katranjiev, one of the editors and now the owners, of *Nova Orbita*, has a favourite joke: "Life is easier for a one-legged man: he only needs one boot". **C.L.**

Attrition in change

Sofia. Nothing changes, but everything is different. People here still shake their heads for "yes" and nod for "no", but what they affirm or reject by so doing has changed. Four years ago, *Nature* reported from Sofia that "... the Red Star atop party headquarters [in Lenin Square in central Sofia] is not going to be pulled down any time soon" (344, 618–620; 1990).

But now the star has gone. The stars have even disappeared from the epaulettes of soldiers and police, to be replaced by the politically neutral rhombus. Lenin's name on all the street-signs in the square has been replaced by that of Baba Nedelya, an historical character of the Renaissance. And people in Bulgaria can no longer be accused of inactivity.

That certainly holds true for the scientific community; 5,000 of the previous 15,000 people connected to the (BAS), have left in the past five years. **C.L.**

The future history of the Solar System

The belief that the Sun will eventually mechanically engulf the orbit of the Earth may be misplaced, but even the coming billion years could bring discomfort to the Earth's inhabitants.

WHEN will the Sun engulf the orbit of the Earth? That was one of a list of questions given, without further discussion, in this year's 125th anniversary issue (*Nature* 372, 14; 1994) as illustrations of our continuing ignorance of the world we live in.

The origin of the question is familiar. The Sun is now a simple and stable astrophysical object, burning hydrogen as a thermonuclear fuel, but the time will come when the helium produced in that process will predominate over hydrogen in the core. Then energy production will be confined to a surrounding shell of hydrogen and the core will itself collapse. At that stage, the Sun will become a red giant star, its vastly extended envelope sustained by an increased flux of radiation, but the external temperature decreased so that it appears redder than now. Red giant stars comparable with the Sun in mass are known to have radii greater than the average radius of the Earth's orbit about the Sun. So is it not meaningful to ask when the Sun's radius will exceed that of the Earth's orbit?

A correspondent, Dr Dieter Hartmann from Clemson University in South Carolina, has now written modestly to say that, while the question is sensible, there is already a cogent answer in the literature. And that is that there is a good chance that the Sun will never engulf the Earth's orbit. The envelope will indeed be enlarged enormously, and the orbit of Mercury will almost certainly be engulfed. But long before that happens, the Sun may have lost up to a quarter of its mass and the Earth's orbit will have been enlarged as the Sun's gravitational attraction declines.

So everything will be all right with the Earth, then? The temptation to jump to that comfortable conclusion will be quickly exorcised by a glance at the article to which Hartmann refers, which is an attempt to chart in detail the behaviour of the Sun over the 10 billion years or more ahead.

The authors are members of a group linked with W. A. Fowler at the California Institute of Technology and comprise I. Juliana Sackmann and Kathleen E. Kraemer from that institution (but the latter has moved to Boston University) and Arnold I. Boothroyd from the University of Toronto (*Astrophys. J.* 418, 457–468; 1993). Their future history of the Sun is the product of a stellar model incorporating the known idiosyncrasies of the Sun, its chemical composition for example.

Predicting the future convincingly requires a demonstration that past history can

be accurately reconstructed. Sackmann and her colleagues are explicit about their assumptions. The age of the meteorites of 4.55 billion years estimated by G. Wasserburg and his colleagues (also at Caltech) presumably marks a point when the early Sun still had a primitive accretion disk, and consisted of a glowing mass of gas and dust whose energy was derived exclusively from gravitational collapse.

That phase, for the Sun, would have occupied no more than 50 million years. For the first 10 million years, as the nebula shrank, the external temperature would have been roughly constant (and roughly 1300 K less than at present) and the luminosity would have declined with the surface area, perhaps by a factor of 30. A further 40 million years would have been occupied in further (and slower) gravitational collapse before hydrogen burning in the core began.

Critical to this model of the Sun are the assumptions made about the original chemical composition of the solar nebula. The raw material of the Sun, consisting as it does of material recycled through earlier generations of stars, contains elements heavier than oxygen produced in supernova explosions, as well as a greater abundance of ^4He than there would have been in the primordial material. Sackmann and her colleagues choose a helium abundance (0.274) that fits with the present luminosity and radius of the Sun. They say that their present use of their solar model is likely to be an improvement on earlier uses of it because they have had the benefit of improved (and higher) estimates of the opacity of the molecular constituents of the outer envelope of the Sun, materials such as CO. The opacity of the stellar material is crucial to determining the extent to which outward radiation pressure sustains the outer regions of the Sun.

What, on this view, is the Sun like at present? The central temperature is 15.43 million degrees and the central density 145.7 g cm^{-3} . Interestingly, after just over 4.5 billion years of hydrogen-burning, the mass fraction of hydrogen at the very centre of the Sun has already fallen by almost a half, from 0.7064 to 0.3632. The radius at which convection takes over from radiative transfer in the outward flux of energy in the Sun is reckoned, from the model, to be 0.741 of the solar radius, at which level the temperature is still nearly 2 million degrees K. Amazingly as always, the fraction of the Sun's mass in this outer convective layer is a mere 1.68 per cent.

And what of the future, as the mass-

fraction of hydrogen at the centre continues to decline? Hydrogen will remain the thermonuclear fuel for the Sun for a long time to come, perhaps for a further 6.4 billion years. But this quiescent phase will not be free from discomfort for those living on the Earth. Over the coming 3 billion years, the luminosity of the Sun will gradually increase by 33 per cent, the external temperature will marginally increase and the radius of the Sun will grow by 13 per cent.

Long before that condition is reached, the Sun's luminosity will have become intolerable. Indeed, there are some who argue that a mere 10 per cent increase of the Sun's luminosity, likely in the next 600 million years, would evaporate surface water. The oceans would disappear when the Sun's luminosity had increased by 40 per cent, perhaps 3.5 billion years from now. Relatively, solar catastrophe is not far away.

Solar cataclysm is not expected until much later. Some 4.8 billion years from now, hydrogen-burning in the core will be halted, and a shell of burning hydrogen enclosing a quiescent core of helium and heavier elements will become thicker over the succeeding 1.6 billion years (taking the luminosity to 2.2 times its present value). That is when the core begins to collapse and when, 6.5 billion years ahead, the Sun's envelope expands rapidly, over 700 million years, to a radius ten times the present.

That is when the fun and games will begin. Now a true red giant, the Sun's radius and luminosity will both increase, the temperature at the centre will have reached 100 million degrees, and helium-burning will eventually begin in a shell surrounding the core. The result of that will be to carry the envelope of the Sun out to the present orbit of Venus, but because the Sun is reckoned to lose 28 per cent of its mass during its red giant phase, even Venus will not be directly engulfed, for the radius of its orbit (inversely proportional to the mass of the Sun) will have enlarged to keep the planet out of range.

The Earth, 38 per cent farther away than at present from the centre of the Sun, will be mechanically safe, but very little imagination is required to guess the effect on its surface of a Sun whose peak luminosity is 2,349 times what it is at present and which subtends an angle of 69 degrees at the Earth's surface. There is worse to follow. In a mere 160 million years, there are five further helium flashes, whereupon the Sun settles down to be a white dwarf and Sackmann and her colleagues "terminate" their calculations. As well they might. **John Maddox**

Ice cores north and south

Jean Jouzel

ONE of the challenges facing palaeoclimatologists involved in the study of glacial-interglacial cycles is to place in a common temporal framework records recovered from terrestrial deposits, deep-sea cores and ice cores. Most crucial is the correlation between deep-sea cores and ice cores because these provide continuous records which contain most of the information about forcings and mechanisms driving quaternary climate change. On page 663 of this issue, Bender *et al.*¹ take an important step towards this objective by comparing the oxygen isotope profiles of two deep ice cores.

Ten years ago, Bender *et al.*² pioneered the analysis of $\delta^{18}\text{O}$ of O_2 in air bubbles contained in ice cores ($\delta^{18}\text{O} = (^{18}\text{O}/^{16}\text{O}) / (^{18}\text{O}/^{16}\text{O})_{\text{SMOW}} - 1$, where SMOW is standard mean ocean water). Their initial interest was to examine the response of the terrestrial and marine biosphere to climate change by documenting the difference between the $\delta^{18}\text{O}$ of atmospheric O_2 and that of sea water. Indeed, Bender *et al.*³ very recently showed that the variability of this 'Dole effect' is small between glacial and interglacial periods, which suggests that relative rates of primary production in the land and marine realms have been fairly constant³. As a result, past variations in $\delta^{18}\text{O}$ of O_2 in air are mainly affected by, and roughly coincide with, variations in the $\delta^{18}\text{O}$ of sea water linked with the waxing and waning of continental ice sheets. This provided a unique tool for correlating deep-sea and ice-core records and allowed Sowers *et al.*⁴ to place records from the Vostok ice core (East Antarctica) and deep-sea sediment cores on a common temporal framework back to 135 kyr before present (BP).

Bender *et al.*¹ have now produced a detailed profile of the $\delta^{18}\text{O}$ of O_2 in the air trapped in the GISP2 core drilled in Central Greenland and extended the Vostok record back to 220 kyr BP⁵. Taking advantage of the fact that this isotopic signal is constant throughout the atmosphere, they use the two records to correlate those two cores. Not surprisingly, this interpretation is divided in two parts. First, there is the period over which various climate records from GISP2 and its sister core GRIP are in excellent agreement (roughly the past 100 kyr). Second, there is the preceding period over which those records diverge, reflecting the alteration due to ice flow of the bottom part of one or both of the cores^{6,7}.

Back to about 100 kyr BP, the correlation between GISP2 and Vostok is straightforward except for the late glacial period where the signal is too flat. This allows examination of how the Antarctic

climate behaves when the Greenland, and more generally the North Atlantic, climates undergo rapid changes. In their new work¹, Bender *et al.* unambiguously demonstrate that interstadial warm periods lasting more than 2 kyr have counterparts in East Antarctica, as previously inferred from visual inspection of the climate records. The correlation between GISP2 and the deep-sea records of $\delta^{18}\text{O}$ of O_2 is also fully exploited. Fortunately, this correlation is more accurate for the early glacial (before 50 kyr BP) when thinning makes the GISP2 varve chronology more

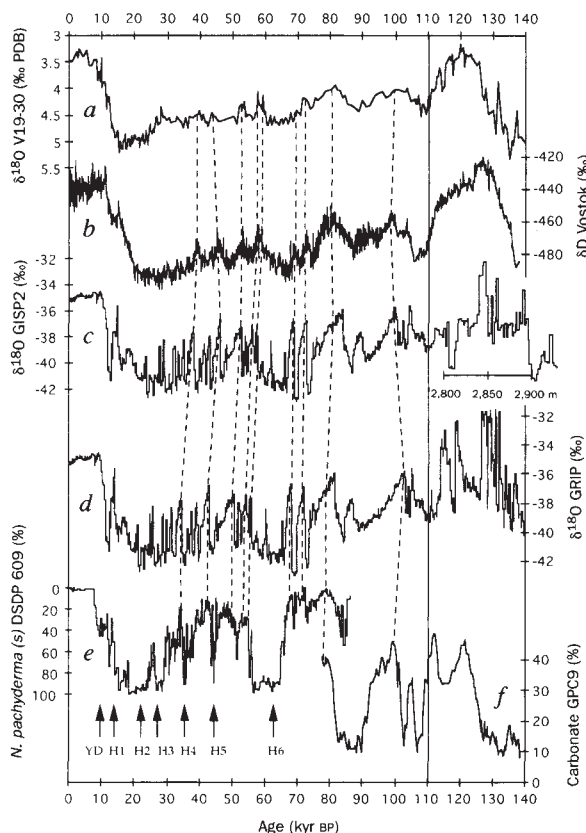
uncertain. Interestingly, the inferred chronology is in excellent agreement with the model derived independently for GRIP⁹, corroborating the assignment of the 110 kyr level in the GRIP core. It is now possible to place any GISP (or GRIP) and marine records on a common timescale. This will allow independent assessment of the relationship between rapid changes as seen in Greenland and in the North Atlantic, which up to now has been based on curve matching^{10,11}.

Before 100 kyr BP, records of $\delta^{18}\text{O}$ for O_2 in air trapped in the GISP2 and Vostok cores no longer covary. As the deepest part of Vostok is still far from the bedrock, the straightforward explanation is that disturbances have affected the bottom part of GISP2. This is not totally unexpected given disturbances in

visible stratigraphy and crystal fabrics previously observed in this core^{6,7,12}.

By itself, this observation of non-correlation does not resolve the ongoing debate¹⁰⁻¹⁶ as to whether rapid changes in climate proxies with depth below 110 kyr BP in the GRIP core reflect rapid climate changes during the Eemian period¹⁷ (which, at least for its later part, does not appear stratigraphically disturbed). However, it suggests that comparison between Greenland and Antarctica is a useful way to examine this problem.

First, in contrast to the earlier period, the ^{18}O and deuterium profiles recorded in the GRIP and Vostok ice are dissimilar during the Eemian. In the absence of a clear climatic explanation for this different behaviour, this adds to the questions raised by the initial comparison of GISP2 and GRIP records⁸. Noticeably, the Vostok interglacial has a short period warmer than Holocene followed by a slightly cooler period. This sequence is similar to recently published records from Germany¹⁵ and the Norwegian Sea¹⁶. Second, measurements of $\delta^{18}\text{O}$ of O_2 in air as a correlating tool between GRIP and Vostok are now under way. GRIP¹⁸ and GISP2 scientists are also using atmospheric composition of methane for the same purpose, hoping that the combination of those two inde-



Ice-core and deep-sea core climate records covering the last glacial-interglacial cycle. The top three curves are adapted from ref. 1. *a*, The benthic $\delta^{18}\text{O}$ record from Vostok 19–30 showing continental ice volume variations. *b*, The Vostok deuterium profile placed on the SPECMAP timescale⁴. *c*, The GISP2 $\delta^{18}\text{O}$ record of ref. 1 extended using a depth scale to illustrate the divergence between GRIP and GISP2 before about 100 kyr BP⁶. *d*, The GRIP $\delta^{18}\text{O}$ record (data and timescale from ref. 9). *e*, The foraminiferal record (a proxy of sea surface temperature) from Deep Sea Drilling Project site 609 placed on the GRIP timescale using the correlations derived in ref. 20. *f*, The weight percentage of carbonate (which likewise reflects changes in thermohaline circulation) in core GPC9 with the chronology defined in ref. 11. The dashed lines drawn between *a*, *b* and *c* of ref. 1 have been extended to GRIP and to the North Atlantic records. The solid line indicates the level at 110 kyr BP (see text). Note that the work of Bender *et al.*¹ in this issue may help to confirm the correlations derived by curve matching between Greenland (*c* and *d*) and North Atlantic (*e* and *f*) climates. Heinrich events²¹ are indicated at the bottom of the plot.

pendent indicators will clarify the stratigraphy below 110 kyr.

More generally, higher-resolution records of both $\delta^{18}\text{O}$ of O_2 and methane trapped in ice cores will shed more light on the correlation between Greenland and Antarctica documented in ref. 1. In this context, the existence of rapid changes in the methane concentration^{19,20} should be extremely useful, in particular for the late glacial. In addition, changes in the methane concentration, an indicator of large-scale variations in the continental biosphere, and small changes in the Dole effect both appear to be affected by the precessional insolation cycle^{3,19}. These indicators may thus provide an independent way to check absolute chronologies and possibly link ice core and continental records. One inherent limitation to the approach is the uncertainty in the difference between the age of the ice and that of the trapped air which may be more than 1,000 years in a low-accumulation site such as Vostok. This uncertainty is lower in higher-accumulation sites such as those of the completed West Antarctic Byrd core and planned drilling sites in both West and East Antarctica.

Thanks to air bubbles trapped in ice cores, we can now hope to know more accurately the leads and lags between Northern and Southern Hemisphere climates and more generally between climate forcings and the various parts of the climate system over the past 100 kyr. This is important not only for understanding past climate changes but also because this period contains information on climate sensitivity and variability which could be the key to our future climate. □

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Faces, fear and the amygdala

John Allman and Leslie Brothers

TWO reports — one on page 669 of this issue¹, the other to appear in *Brain*² next February — provide rare and remarkable evidence that in humans a brain structure called the amygdala participates in the perception of social signals. Both groups find that damage to the amygdala produces selective deficits in the perception of facial expression. The amygdala is roughly the size and shape of an almond nut, and lies deeply buried in the temporal lobe.

Adolphs and colleagues¹ studied a patient (S.M.) who had suffered destruction of the amygdala as a consequence of Urbach-Wiethe disease. In this condition, the amygdala in both hemispheres is nearly completely destroyed by the deposition of calcium while the hippocampus and neocortex are spared. S.M. is unable to discern fear in facial expression, and is unable to discriminate between fine differences in other facial expressions that are readily perceived by normal subjects. Young *et al.*² studied patient D.R., who has partial lesions in the amygdala on both sides as a consequence of treatment for epilepsy. D.R. is very poor at matching photographs depicting facial expressions to either the name of the emotion portrayed or to other photographs illustrating the same emotion; this patient also has great difficulty in telling whether individuals are looking at her or away, which normal subjects do with ease.

These two papers add substantial support to the growing evidence from animals that the amygdala has a central role in social communication. In the 1950s it was discovered that light touch to the skin could be an extremely potent stimulus for driving amygdala neurons in the cat³; flank-rubbing is an important means of affiliative signalling in cats. So this finding lends itself to the retrospective interpretation that the investigators had stumbled upon a social function in the cat amygdala. Indeed, subsequent single-neuron recording in the amygdala of freely moving cats showed that "miaowing was the most effective of all sensory stimulation tested"⁴.

The idea that there is a specific neural substrate for social cognition in primates was first proposed by Kling and Steklis⁵. From their studies of the effects of brain lesions on social behaviour in monkeys,

they proposed that a system including the amygdala, temporal pole and orbital frontal cortex underpins social ties in monkey groups. In monkeys, neurons in the superior temporal sulcus, an area connected to the amygdala, are sensitive to images of faces as well as to the direction of gaze and orientation of the faces^{6,7}; and lesions in the superior temporal sulcus produce a selective deficit in the capacity to discriminate the direction of gaze in face

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Fearful features, from nineteenth-century France.

images⁸. Neurons of the amygdala are similarly sensitive to the direction of gaze⁹.

In humans, it could be that certain conditions stem from defects associated with the amygdala. For example, autistic children are inattentive to facial expression, and it seems that they fail to interpret gaze direction normally^{10,11}. It is possible that the interactional problems typical of autism arise from dysfunction in this neural system that converges in the amygdala. It is also possible that abnormal activity of the system could yield the distorted perception of social signals that is typical of the paranoid delusional state.

The direction of one's gaze signals the object of one's attention (that is, what one 'has in mind') while facial expression indicates how one is disposed to behave. When mutual eye contact is established,

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both participants know that the communication loop between them has been closed, and for primates of all species this is the most potent of social situations. The discovery that the human amygdala is involved in detecting both gaze direction and facial expression shows that it is indeed part of a brain mechanism for representing the intentions and dispositions of others. But we have a long way to go before we have a complete picture of the human amygdala's responsiveness. Could the amygdala also be essential for the interpretation of social touch and voice intonation — or even the sounds of footsteps? Studies on the monkey amygdala which used a wide range of social stimuli found single neurons that responded to dimensions of the social environment ranging from body movements to interactions taking place between other monkeys^{9,12}.

From animal experiments there is considerable evidence that the amygdala plays a crucial role in fear conditioning, and indeed the amygdala projects to the structures in the hypothalamus and brain stem that regulate the autonomic expressions of fear such as increased heart rate and sweating¹³. Damasio¹⁴ (one of the authors on the *Nature* study¹) has proposed that the amygdala and orbital frontal cortex participate in decision-making by correlating somatic states, such as heart rate, with behavioural situations; that is, individuals choose to avoid situations that are associated with a negative outcome marked by 'gut feelings'. It would be extremely interesting to know whether patients with damage to the amygdala are defective in fear conditioning and decision-making abilities. □

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TRANSLATION

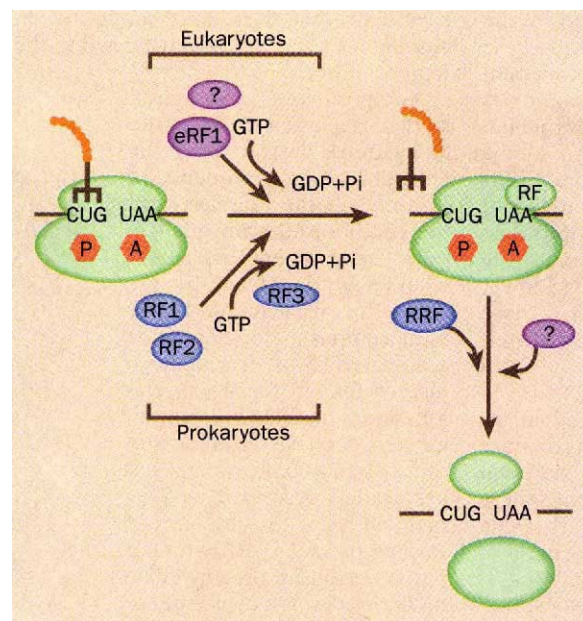
Knowing when to stop

Mick F. Tuite and Ian Stansfield

TRIPLET signals built into a messenger RNA's coding sequence tell the ribosome when to stop translating that mRNA. These are the so-called stop codons UAA, UAG and UGA, and in bacteria they signal the termination of mRNA translation by facilitating the binding of polypeptide release factor to the ribo-

purify their eRF, termed eRF1, was originally developed to identify the soluble components of the translation termination machinery of *Escherichia coli*³. Three such components have now been found in *E. coli*: RF1 and RF2, which catalyse polypeptide-chain release at UAA/UAG and UAA/UGA codons respectively^{3,4}, and RF3, which stimulates RF1 and RF2 activity in a GTP-dependent and codon-independent manner⁵. The original studies on the eRF, using the same biochemical assay, showed that a single rabbit eRF will catalyse polypeptide release in response to all three stop codons also in a GTP-dependent manner². The omnipotence of the termination activity of the eRF, together with an almost complete absence of sequence similarity between the newly identified eRF1 family and the bacterial release factors, may indicate a fundamental mechanistic difference between the eukaryotic and bacterial processes.

There has been one false start in the race to identify the eRF by molecular cloning. This was in 1990, with the report⁶ of the cloning of a complementary DNA encoding the putative rabbit eRF which showed a significant (more than 90%) amino-acid identity to



Polypeptide chain release from the ribosome in prokaryotes and eukaryotes requires one or more release factors (RF) and GTP. Following polypeptide release, the ribosome dissociates from the mRNA with the help of a ribosome release factor (RRF), the identity of which is unknown in eukaryotic cells. Release-factor activity for a given protein is assayed *in vitro* by determining the ability of the protein to release a radioactively labelled peptidyl-tRNA from the ribosomal P site in response to a stop codon occupying the ribosomal A site.

some, thereby stimulating hydrolysis of the final peptidyl-transfer RNA linkage and releasing the completed polypeptide chain from the ribosome (see figure). The same three stop codons are used in eukaryotic mRNAs, but exactly how the codon signals are transmitted to the eukaryotic ribosome has remained something of a mystery.

Until now, that is, for on page 701 of this issue¹ Frolova *et al.* report the identification of a family of eukaryotic polypeptides with the biochemical properties expected of a eukaryotic release factor (eRF). The existence of a protein with release factor activity in eukaryotes became apparent following its biochemical demonstration, some 20 years ago, in rabbit reticulocytes². So the subsequent identification of the eRF, and of the gene encoding it, has taken a surprisingly long time.

The assay employed by Frolova *et al.* to

tryptophanyl-tRNA synthetase (TrpRS), the enzyme used to catalyse the addition of tryptophan onto the mammalian tryptophanyl-tRNA (Trp-tRNA) in an ATP-dependent reaction. But there was a problem — how could this specific, ATP-dependent, tRNA-charging enzyme be required to terminate translation when its usual substrate, Trp-tRNA, can only decode the UGG (Trp) codon? The dilemma was finally resolved last year when it was demonstrated unequivocally that purified mammalian TrpRS does not itself have release-factor activity, but rather copurifies with a protein that does⁷. That protein, we now know, is the eRF1 polypeptide.

Frolova *et al.* exploited their ability to separate release-factor activity from the TrpRS activity, and used protein sequencing to show that the polypeptide with release-factor activity had significant sequence similarity with a ribosome-

associated protein originally identified in the yeast *Saccharomyces cerevisiae*. This yeast protein, variously designated as Sup45, Sup1 or Sal4, has been implicated in translation termination through biochemical studies and genetic analysis of mutants affecting the efficiency of non-sense suppression *in vivo* (reviewed in ref. 8). Frolova *et al.* have not demonstrated that the yeast Sup45 protein has release-factor activity. However, given that it shows 68% amino-acid identity to its higher eukaryotic counterparts^{1,9}, and that a mutation in the *SUP45* gene can be complemented by the *Xenopus* Sup45 homologue Cl1 (ref. 9), it may well serve the same function in translation termination.

Is eRF1 the only release-factor required for efficient and accurate translation termination *in vivo*? We would suggest that this is unlikely, not least because *in vitro* studies demonstrate a GTP requirement for the rabbit release-factor activity, yet no members of the eRF1 family appear to contain a consensus GTP-binding site^{1,9}. One candidate for an additional component of the eukaryotic translation termination machinery might be the Sup35 protein. Originally identified in yeast, mutations in the *SUP35* gene show many of the phenotypes associated with a defect in termination (reviewed in ref. 8), and there is also genetic evidence that the

Sup35 and Sup45 polypeptides functionally interact¹⁰. Consistent with it providing the GTP requirement for termination, the Sup35 polypeptide contains several consensus GTP-binding sites¹¹. Given that Sup35p may be a prion-like protein able to take up two functionally distinct conformations which differentially influence the efficiency of translation termination^{12,13}, we have the tantalizing possibility that translation termination in eukaryotes may be controlled in a similar way. □

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ANTIGEN PRESENTATION

Chewing the fat

Peter Parham

A FUNDAMENTAL principle of the human immune system is that anything not perceived as self is defined as foreign. By this rule, cytotoxic T cells are selected not for their skill in responding to the foreign peptides that signal virus infection or nascent malignancy, but for the compatibility of their interaction with self-peptides bound to polymorphic class I HLA-A,B,C molecules. Although it is somewhat bumbling, there is logic to this strategy where self-preservation scores above humbling an invader. Above all it is flexible, enabling some sort of response to be made to any new or unanticipated foe. It also seems to provide a foundation upon which smarter tactics, targeted to particular groups of foreigners, can be overlaid, as illustrated on page 691 of this issue¹. There Michael Brenner and co-workers report on experiments implying that CD1b, a monomorphic class I molecule, specializes in presenting certain lipid antigens to cytotoxic T cells. These lipids, the mycolic acids, are characteristic of those mycobacteria that cause tuberculosis.

Like the more familiar HLA-A,B and C

molecules, CD1b consists of a class I heavy chain associated with β_2 -microglobulin. But the CD1b and HLA-A,B,C heavy-chain sequences are so divergent that similarities are detected only at the protein, not the DNA, level; the CD1b heavy chain gene resides not in the major histocompatibility complex (HLA region) on chromosome 6 but is one of five CD1 genes on chromosome 1, which are neither polymorphic nor show the ubiquitous tissue distribution of HLA-A,B and C. Instead CD1 molecules are expressed on cortical thymocytes and various 'professional' antigen-presenting cells, including dendritic cells, intestinal epithelium and the Langerhans cells of skin epidermis^{2,3}. Such properties intrigue immunologists and raise questions as to the part played by the various CD1 molecules, divergent also amongst themselves, in antigen presentation. T cells recognizing CD1 molecules have been sought and found to be unconventional^{4,5}. They express neither CD4 nor CD8 co-receptor: the DN1 line studied by Beckman *et al.*¹ is an $\alpha\beta$ T cell; others are of the $\gamma\delta$ lineage⁵.

Derivation of DN1 required the wizardry of the modern cellular immunologist. T cells lacking CD4, CD8 and the $\gamma\delta$ T-cell receptor were isolated from blood and stimulated with dried, disrupted *Mycobacterium tuberculosis* in the presence of monocytes (the antigen-presenting cells) induced by co-culture with cytokines to express CD1 molecules. To favour outgrowth of T cells targeted towards monomorphic CD1 molecules and not polymorphic HLA-A,B,C, the T cells were stimulated successively with antigen presented by the monocytes from individuals with different HLA. The resulting DN1 line has cytotoxic activity, specific for target cells which express CD1b and have been exposed to the antigenic extract⁴.

Using some vintage biochemistry, Beckman *et al.* have hunted down the antigenic principle of the crude mycobacterial extract. Resistant to protease and soluble in detergent, it partitions with the organic phase on extraction with chloroform/methanol. Such are the properties of a lipid and further fractionation showed the antigen co-purified with the mycolic acids, long-chain β -hydroxy fatty acids which are characteristic components of the mycobacterial cell wall⁶.

Homo sapiens has neither cell walls nor mycolic acids. These entities are alien to the human cell and its guardian the thymus-derived lymphocyte. In medical practice the smartest antibiotics are those which perturb a unique aspect of bacterial metabolism and leave the host alone. Unexpectedly, CD1b seems to be a component of our immune system that has cottoned on to the same strategy. In surveying our tissues for trauma, the common T cell has the unenviable job of identifying small numbers of foreign peptides from amongst a sea of chemically similar self-peptides presented by the very same HLA-A,B or C allele. By comparison, discerning the presence or absence of a mycolic acid on a CD1b molecule would seem to be a simpler, clear-cut task for the DN1 T cells.

A molecule which presents mycolic acid, presumably causing T cells to harangue mycobacteria, must surely be a good thing. Indeed everybody has one. Mice, on the other hand, appear not to have made the investment, because their pair of CD1 genes does not include one of the CD1b isotype. This seemingly quirky difference actually illustrates a principle emerging from the study of non-polymorphic, antigen-presenting class I genes. These genes are not always common to mammalian species and their functions appear to represent innovations developed after the separation of the mammalian orders. For example, mice but not humans possess a class I molecule that binds peptides having amino-terminal

N-formyl methionine at their amino terminus and is therefore specialized for presenting peptides derived from bacterial proteins⁷. Unfortunately, this desirable function is not yet on offer to the human consumer.

In contrast the monomorphic class I molecule (FcRn), which works as an Fc receptor to provide the very young with maternal IgG, is shared by humans and mice⁸. Although it is similar in structure to the peptide-presenting class I molecules⁹, FcRn does not bind peptides. Nor does it bind to the Fc of immunoglobulin G in a manner analogous to that envisaged for the interaction between T-cell receptors and class I molecules¹⁰. Clearly there is functional versatility in the class I structure and CD1 may turn out to be different again. That humans lacking the peptide transporter have low HLA-A,B,C levels but normal CD1a expression¹¹ argues against this molecule needing to bind peptides. Indeed one wonders if self molecules, lipids or otherwise, are ever chewed upon by CD1 molecules and whether the particular interaction of CD1b with mycolic acid uses the 'peptide-binding site', which even for HLA-A,B,C is a generally greasy groove.

Examination of the antigens recognized by T cells is an increasingly sophisticated and chemically orientated business. Immunologists, who just a few years ago were happily watching T cells respond to crude digests of sperm whale myoglobin or transplanted tissues of another species, suddenly found themselves going through a crash course in the synthesis, separation and study of short peptides. Now, just when they are becoming comfortable with high-pressure liquid chromatography, mass spectrometry and protein crystallography, the ghastly message of Beckman *et al.* is that all of this may not be enough. The future might lie with the lipids. To ensure a place on the fast track the most modern immunologist now needs to fret about fat. □

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February's bizarre event

Peter J. T. Leonard

IN the 1960s, military reconnaissance satellites designed to detect the explosions of nuclear weapons discovered what remains perhaps the most baffling phenomenon in all of astrophysics: isolated bursts of gamma-rays, each lasting only seconds, and appearing at the rate of about one per day at a random location on the sky. Early this year, the most remarkable burst yet was detected. As Hurley *et al.* describe on page 652 of this issue¹, the event lasted over an hour and included photons of energies up to 18 GeV, the hardest ever seen in such a burst.

The origin of γ -ray bursts (GRBs) remains a mystery. Thus far, they have been detected only in γ -ray and X-ray regions of the electromagnetic spectrum, and have not been linked to any known object in the Universe (see for example ref. 2). The physics behind GRBs is a challenge to understand, as the spectra are highly non-thermal and the luminosity fluctuates rapidly. It was hoped that BATSE (the Burst and Transient Source Experiment), EGRET (the Energetic Gamma-Ray Experiment Telescope) and COMPTEL (the

Compton Telescope) on board the Compton Gamma-Ray Observatory (CGRO) would solve the mystery, but their results have only deepened it. The first important CGRO results, established by BATSE, are that the GRB sources are distributed fairly uniformly on the sky, and their spatial distribution is finite³. These results taken together rule out distant sources confined to the Galactic disk and nearby sources in the Galaxy.

The high-energy, long-duration burst observed by CGRO on 17 February 1994 may turn out to be as serious a constraint on the theory as the earlier CGRO discoveries. This event was not very unusual as observed by BATSE, but was quite bizarre as seen by EGRET, which detected several gigaelectronvolt photons, including one with an energy of 18 GeV that arrived 80 minutes after the original burst. Annoyingly, there was an intervening one-hour period when the Earth was between the source and CGRO, and it is not known whether continuous emission of gigaelectronvolt photons occurred during this interval.

Meteorite made with a fizz

IMAGE
UNAVAILABLE
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REASONS

M. M. Grady, Natural History Museum

THESE seemingly innocuous orange grains of carbonate (each 100–200 μm across) may help answer the question of how warm and wet Mars was in its early history. They are not, of course, ordinary terrestrial carbonates. Rather, they were found embedded in a meteorite that was once a piece of the martian crust, and they thus provide a record of fluid-rock interactions on ancient Mars.

On page 655, C. S. Romanek *et al.* report measurements of the oxygen and carbon isotope compositions of the carbonates that place important constraints on their formation conditions. Whereas an earlier reconnaissance study hinted at formation temperatures as high as 700 °C (suggestive of hydrothermal processes at depth — see *Nature* 369, 356; 1994), the new oxygen isotope measurements provide evidence that the carbonates precipitated in a cooler environment (temperatures in the range 0 to 80 °C) from water rich in dissolved CO₂. Moreover, the carbonates are richer in ¹³C than their terrestrial counterparts, consistent with CO₂ in the martian atmosphere being the source of carbon. In other words, it now appears that warm, fizzy soda water was once percolating through the near-surface crust of Mars.

Karl Ziemelis

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Rumours of the February 17 event spread rapidly, and two explanations for understanding it within the framework of the popular model of merging pairs of neutron stars in distant galaxies were quickly forthcoming. Assuming that the initial GRB occurs during a merger between two neutron stars, a second burst might occur when the highly relativistic ejected material strikes a dense ring of material that was thrown off much earlier in the evolution of the merging binary⁴. Alternatively, the delayed emission might occur when the merger ejecta slams into interstellar matter located far from the merger site⁵. However, the production of photons with energy greater than 10 GeV remains a challenge in both cases.

The February 17 event might also be interpreted as a gravitationally lensed GRB at a cosmological distance. In this case, the two bursts of photons observed would have followed paths that differed in length by more than one light hour. This hypothesis sidesteps the issue of the physics of GRB sources at cosmological distances, and appears unlikely, as gravitational lensing does not alter the spectrum of the source, whereas the 'second' event here had a much more energetic spectrum than the first. In fact, it was not seen at all by BATSE or the GRB detector on board the Ulysses spacecraft.

Many astrophysicists still find it desirable to have the GRB sources within our Galaxy as opposed to lying at cosmological distances, as the physics behind their non-thermal, non-monotonic properties would be far easier to imagine. At present, the main hope for a Galactic distribution of sources is a very extended halo. Recently considered are GRB sources that are ejected from our Galaxy at high speeds, continue to burst for at least 10^9 years, and are gravitationally perturbed by the galaxy M31 (ref. 6). These conditions result in a very extended and well mixed distribution of sources that mimic the observed isotropy and radial cut-off.

To my mind, the high energies and long duration of the burst on February 17 might be explained by the accretion of an asteroid onto a high-speed Galactic neutron star⁷. In this model, the neutron star has a magnetic dipole of typical strength (10^{12} to 10^{15} gauss), and is rotating too slowly to be observable as a pulsar. The angular momentum of the infalling asteroid twists the magnetic field lines so much that their eventual reconnection accelerates electrons and ions to high energies⁸. The electrons radiate their energy promptly as a GRB. It is plausible that the ions stored in the neutron star's magnetosphere reach far higher energies than the electrons, and ultimately produce gigaelectronvolt photons long after the initial event.

Observations of other GRBs like the February 17 event would help to narrow

down the theory. With luck, the next such GRB will occur near one of the poles of CGRO's orbit, so that the Earth will not get in the way, and we might find out whether the emission of gigaelectronvolt photons was continuous during the missing hour. Also, the discovery of such an event in progress might provide time enough for Earth-based teraelectronvolt detectors to turn towards the source to search for unattenuated γ -rays. Teraelectronvolt photons from cosmological sources are unlikely to reach the Earth, as they have a high probability of interacting with the cosmic microwave background, whereas such γ -rays from Galactic sources would not experience this attenuation. If teraelectronvolt photons are detected, this would suggest that GRB sources do not lie at cosmological distances.

There are at least three additional observations that would help to resolve the GRB controversy. First, if the repetition of GRBs^{9,10} turns out to be genuine, then it would argue against the theory of a neutron-star merger, as such events cannot repeat. Better BATSE positions are required to rule on this¹¹. Second, measuring the column densities to GRBs at soft X-ray wavelengths may reveal the relevant distance scale¹². Afterglows from GRBs have been detected at such wavelengths¹³. Third, if GRB sources lie at cosmological distances, then a dipole

anisotropy will eventually show up in their distribution on the sky, just as is the case for the cosmic microwave background. It is estimated that about 10^4 BATSE bursts will be required to reveal this¹⁴. Of course, some hint of an excess of sources towards the Galactic Centre or the galaxy M31 may show up first, which would argue in favour of extended-halo models for the bursts. □

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CATALYSIS

What promise for dendrimers?

Donald A. Tomalia and Petar R. Dvornic

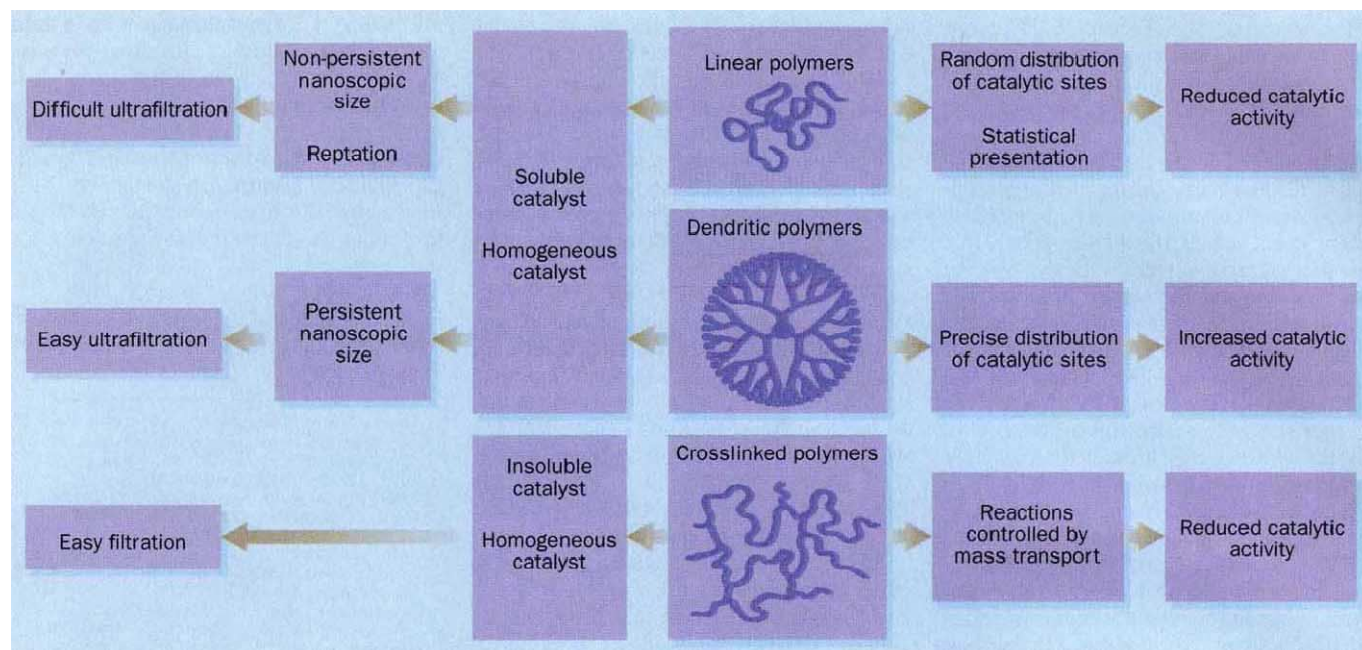
WHAT potential could precise macromolecular fractals¹, constructed by geometrically progressive structural branching, possibly offer to the scientific and industrial community? This is a question that has been asked many times over the past decade, since the discovery of the new class of polymeric architecture called dendrimers². Would these tree-like molecules initiate new fields of application and research or would they merely be remembered as esoteric objects of symmetrical beauty? On page 659 of this issue, a group led by Gerard van Koten³ suggests the former, by describing the first example of a catalytic dendrimer with active metal sites residing on the outer surface of the snowflake-like structures.

The species that Knapen *et al.*³ have prepared is a well characterized, soluble polycarbosilane dendrimer^{4,5} with diamino arylnickel(II) complexes presented as precisely ordered groups on the surface of this nanoscale scaffolding. As true multi-metallic species, these compounds assisted in addition reactions of polyhaloalkanes to double bonds, with catalytic

activity and regiospecific formation of 1:1 addition products comparable to that observed for the monometallic complexes.

The hope is that these dendrimeric catalysts will retain the benefits of homogeneous catalysts (faster kinetics, accessibility of the metal site and so on) but that, unlike most such species, they will be readily recoverable after reaction. The large (nanoscopic) size and relative rigidity of dendrimers should allow them to be removed from solvent streams by ultrafiltration methods (see figure).

Classical polymers have exerted tremendous influence on the field of chemical reaction catalysis. Polymeric catalysts, which consist of conventional catalytic species anchored to a polymer carrier, have the advantage that they are usually considerably less corrosive and more stable, active and selective than their mononuclear counterparts. Depending on whether the polymer carrier is linear or crosslinked, the polymeric catalyst may be soluble (allowing homogeneous catalysis) or insoluble



The benefits of branching — schematic illustration of the three main types of polymer catalysts, together with their distinguishing properties and functional characteristics. The properties to the left of the

structural sketches result from the physical and chemical characteristics of the polymer carrier, whereas those to the right result from the locations of catalytic sites.

(heterogeneous catalysis). The latter has the advantage that it can be easily separated by filtration from the product reaction mixture, but the disadvantage is that reactions taking place on the interior surface of a porous catalysis particle encounter resistance to mass transport through the pores.

The performance of a homogeneous polymeric catalyst is determined by the physical and chemical properties of the catalytic sites, by the distribution of these sites along that polymer backbone, and finally by the accessibility or presentation of these sites to the reacting substrates. Under given reaction conditions, these factors are usually governed by the polymer chain conformation, which in turn depends on the reaction solvent and temperature. The rate of catalysis depends on the rate of diffusion of both substrates and products to and from the catalytic sites. Therefore, the more densely coiled the catalyst-bearing polymer backbone, the less accessible are the catalytic sites, and usually the more inefficient the overall catalytic process. Consequently, an ideal polymeric catalyst should be a soluble, multifunctional macromolecule, favouring configurations in which all active sites would always be exposed towards the reaction mixture so that they were easily accessible to migrating reactants and unlikely to be hindered by catalytically inactive components of the carrier molecules.

In addition, the polymer scaffolding should show all the advantages of insoluble catalysts, particularly in ease of separation from the resulting reaction-product mixtures. For this reason, the ideal polymeric catalyst should be sub-

stantially different in size from the substrates or reaction products and be persistent in that difference — it should not, for instance, be able to change its hydrodynamic volume by reptating through a filtration barrier.

The authors have not yet directly compared the catalytic activity of these dendrimers against their linear and cross-linked counterparts, so they cannot yet set numbers on the anticipated advantages discussed above. Nonetheless, there is an impressive list of demonstrated physical and chemical properties attributed to dendrimers that clearly differentiate them from classical polymer systems. The list^{6,7} includes persistent and controllable nanoscale dimensions (1–100 nm); controlled shape design based on the choice of core unit; precise masses (with polydispersities of $M_w/M_n \approx 1.0005$); chemically reactive surfaces (over 100 surface reactions and modifications have been demonstrated); interiors that could be specifically designed to be suitable for hydrolytically or thermally demanding environments (over 30 different dendrimer interior compositions are known); and designable solvent solubilizing surfaces (hydrophobic or hydrophilic types).

These properties, or some combination of them, are what make dendrimers so useful in such non-catalysis applications as nanoscale reactors^{8,9}, micelle mimicry^{10,11}, magnetic resonance imaging agents¹², immuno-diagnostics¹³, gene delivery vectors¹⁴, nano-antennae¹⁵ and nano-scopic building blocks for more complex megamolecular structures¹⁶.

At present, classical polymer architectures are used in a wide variety of catalytic applications such as classical

organic synthesis, cracking and isomerization of hydrocarbons, nitrations, esterification hydrolysis and decarboxylations, and as supported transition-metal catalysts and polymerization initiators, to mention a few. Now Knapen *et al.*³ have opened a new avenue by introducing these first dendrimer-supported catalysts. With the possibility of combining catalysis with other unique features of dendrimers, expectations are running high in this area. Only time and more experimentation will determine whether dendrimer catalysts will live up to their generous promises. □

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Endogenous cannabinoids

Leslie Iversen

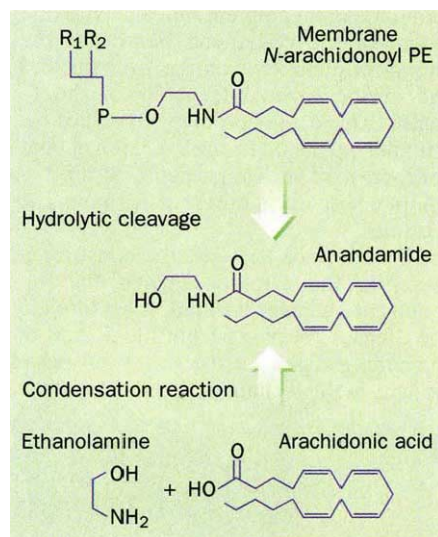
TAKEN together with two other papers^{1,2}, the report by Di Marzo *et al.* on page 686 of this issue³ will excite controversy about the mechanisms involved in the biosynthesis and physiological disposition of the brain lipid anandamide (*N*-arachidonoyl-ethanolamine). The reason for the interest in this substance is that it is thought to represent the endogenous cannabis-like substance in brain. Knowledge of the mechanisms involved in the metabolism and inactivation of anandamide could point to fresh approaches to the pharmacological manipulation of these systems in brain, and so to potential therapeutic applications.

The psychoactive component of marijuana, Δ^9 -tetrahydrocannabinol (THC), acts by binding to a specific receptor in brain^{4,5}. The brain cannabinoid receptor belongs to the G-protein-coupled superfamily, and when activated inhibits adenylate cyclase activity and voltage-sensitive calcium channels of the N-channel subtype^{6,7}. The discovery of a receptor in brain that specifically recognizes Δ^9 -THC and related synthetic cannabinoids pointed to the existence of an endogenous ligand, and the most plausible candidate is anandamide⁸. So far, little is known about the mechanisms involved in the synthesis, release and inactivation of this putative new neural signalling molecule. The three new papers¹⁻³ address some of the relevant questions, although that by Di Marzo *et al.* comes to quite different conclusions to those reached by the others — particularly about the mechanisms involved in the biosynthesis of anandamide in brain.

Devane and Axelrod¹ and Kruszka and Gross² propose that anandamide is formed by the enzymically catalysed condensation of arachidonic acid with ethanolamine. Both laboratories demonstrated the formation of anandamide when brain membrane preparations were incubated *in vitro* with ethanolamine and arachidonic acid. Kruszka and Gross suggest that the condensation reaction is unlike others involving arachidonic acid in being independent of coenzyme A and ATP. Instead they propose that arachidonic acid may react with a cysteine at the active centre of the enzyme to form an arachidonoyl-thiol ester enzyme intermediate. Both groups found that arachidonic acid was the preferred substrate among a series of related unsaturated fatty acids tested, and that the reaction was specific for ethanolamine.

Di Marzo and colleagues³ put forward an alternative biosynthetic mechanism. They propose that anandamide may be synthesized by hydrolytic cleavage from

a pre-existing phospholipid precursor, arachidonoyl phosphatidylethanolamine (APE; see figure). Although they identified no specific synthetic enzyme, phospholipase D was shown to be capable of forming anandamide from endogenous brain lipid fractions containing the proposed precursor. In primary cultures of rat brain neurons exposed to radiolabelled ethanolamine, Di Marzo *et al.* showed that stimulation with the calcium ionophore ionomycin (to simulate synap-



Alternative routes for the biosynthesis of anandamide.

tic activity) led to the formation of radiolabelled anandamide. Furthermore, the authors demonstrated that the newly synthesized anandamide is released into the external medium, and that it can be inactivated by a rapid, saturable uptake mechanism. Incubations using neuron-free astrocyte cultures did not lead to anandamide formation, indicating that its biosynthesis was restricted to neurons. Further experiments, using chromatographic and nuclear magnetic resonance techniques, established the presence of the hypothetical precursor phospholipid APE in brain lipid extracts.

Di Marzo *et al.* argue against the hypothesis that anandamide biosynthesis involves a condensation reaction, on the grounds that the endogenous levels of free arachidonic acid and ethanolamine in brain are very low. In the experiments^{1,2} in which anandamide was formed by the condensation route, the reaction may have been driven artificially by the use of very high substrate concentrations (20–100 mM in the case of ethanolamine), and it is suggested that in reality these are enzymic routes for the breakdown of anandamide being driven in reverse.

On the other hand, under the conditions used by Di Marzo *et al.* only very small amounts of radiolabelled ethanolamine were incorporated into anandamide following stimulation of neuronal cultures. Larger amounts of other *N*-acyl-ethanolamines were formed, including *N*- γ -linolenoyl, *N*-linoleoyl, *N*-palmitoyl and *N*-stearoyl, implying that the biosynthetic mechanism proposed is rather unspecific. The authors counter this by arguing that this finding may suggest that in the brain there is a family of *N*-acyl-ethanolamines with differing cellular signalling functions. This is an interesting proposal which deserves further attention. Is it possible, for example, that another *N*-acyl-ethanolamine could be the endogenous ligand for the second cannabinoid receptor described in peripheral tissues^{9,10}, for which anandamide has only modest affinity?

Regardless of the outcome of the debate on biosynthetic mechanisms, Di Marzo and colleagues have advanced our understanding of anandamide as the putative endogenous cannabinoid receptor ligand. Their findings suggest that anandamide biosynthesis is under physiological regulation, being stimulated by increased neuronal intracellular levels of calcium, and that anandamide can be released into the extracellular medium and recaptured by an uptake mechanism.

In a more cautious vein, it is worth noting that not everyone agrees that anandamide is the endogenous cannabinoid receptor ligand in brain. For example, Evans and colleagues¹¹ also reported the release of endogenous cannabinoid-receptor-binding activity from rat brain slices *in vitro* in response to depolarizing stimuli or to calcium ionophores. In this case, however, the material released appeared to be an unidentified peptide and not anandamide, so this story may still be far from complete. □

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Silence speaks in spectrin

Walter Gratzer

All llamas and camels, but only about 4 in every 10,000 people, have elliptical red cells. In people (but not in camels) such cells are generally the mark of a haemolytic anaemia. The inheritance is nearly always dominant, and the condition is often lethal in homozygotes. It has long irked geneticists that heterozygotes, even within one family, may be in vigorous health and unaware of their ailment, or may be transfusion-dependent and sometimes dead by the age of puberty.

More than 40 years ago Motulsky conjectured that a second genetic defect, normally silent, might be behind this seeming affront to the principles of Mendelian genetics. Two laboratories, one in Lyon and one in Paris, have made much of the running in pursuit of an explanation for these phenomena, and they have now come up^{1,2} with a satisfyingly complete structural account of the variations of phenotype, rather along the lines prefigured by Motulsky.

A rich variety of mutations that lead to hereditary elliptocytosis have been identified. Most often they are in spectrin, the

filamentous protein that makes up the elastic links of the submembranous lattice (the membrane cytoskeleton) on which the mechanical stability of the red cell depends. Spectrin contains two types of polypeptide chain, both composed of a series of structural repeating units in the form of triple-stranded α -helical coiled-coils. The structural member of the membrane skeletal network is a tetramer, formed by the head-to-head association of two antiparallel $\alpha\beta$ -dimers. Both chains terminate in incomplete repeats, with one strand of the coiled-coil bundle at the amino-terminal end of the α -chain and two at the carboxy-terminus of the β -chain. These — as the properties of mutants suggested, and Speicher and colleagues³ in due course demonstrated — complement each other in forming the tetramer.

This interface between the constituent dimers of the tetramer is where the mutations are nearly all found, most often in the α -chain (deriving from the 5' end of the coding region of the gene) but occasionally in the β -chain (stemming from the

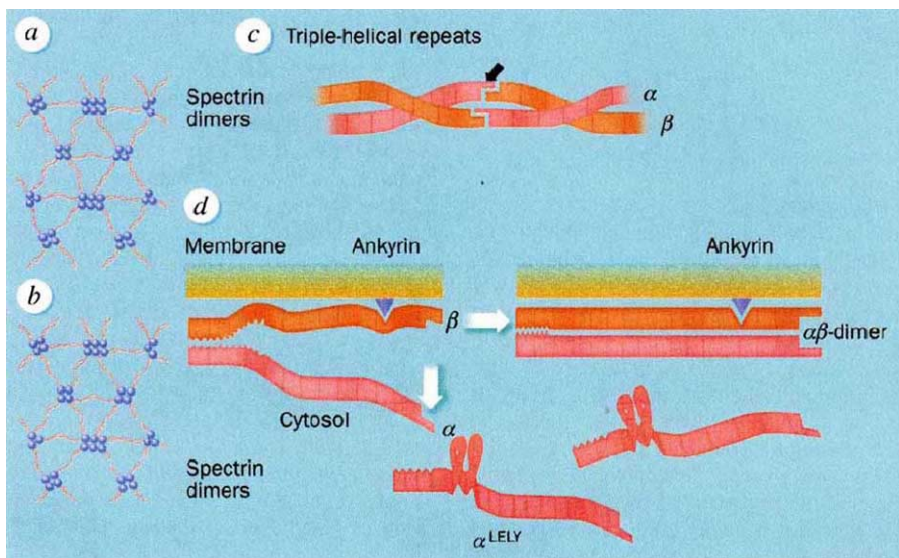
3' end of the coding sequence). Parquet *et al.*¹ describe the discovery of three new mutants, one in the α -chain and two in the β -chain, all of which disturb the dimer-dimer association (though to somewhat different extents).

Clinical severity correlates with the proportion of spectrin dimers that fail to form tetramers and thus disrupt the continuity of the network. This in turn depends on the proportion of the spectrin mutant in the assembly; but why does this expression of the defective protein vary so much from one victim of the mutation to another? Well, Randon *et al.*² have uncovered the ingenious answer.

Earlier work by Delaunay and his colleagues at the Lyon laboratory had shown that there is a common, but low-expression and symptomless spectrin polymorphism — one-third of all *Nature* readers have it — and identified it as a substitution towards the far end of the α -chain; the mutation is designated as α^{LELY} (low-expression allele, Lyon). To understand why it is apt to be calamitous only for carriers of a mutation for hereditary elliptocytosis, we need to recall something of the sequence of events during erythropoiesis.

A decade ago, Moon and Lazarides⁴ established that the β -chain is synthesized at an early stage of assembly and binds to its attachment site (ankyrin) on the membrane. Thereafter the α -chain floods the system and the part not taken up into an equimolar complex by the β -chain is destroyed by proteases in the cytosol. More recently, Speicher *et al.*⁵ worked out the mechanism of the lateral association of the constituent chains of the spectrin dimer. It turned out that strong interactions between pairs of repeats along the α - and β -chains are confined to four contiguous repeats at one end of the dimer. These bind strongly to one another and the remaining repeats then presumably pair off cooperatively, like a DNA duplex zipping up from a GC-rich nucleation site.

Randon *et al.* have found that it is one of these critical repeats that is perturbed by the α^{LELY} mutation. Consequently, when the freshly synthesized α -chains are assimilated by the β -chains already on the membrane, the normal α -chain is preferred to the mutant. With such a high frequency in the population of the α^{LELY} gene, a third of the heterozygotes carrying an elliptocytosis mutation at the end of the spectrin α -chain will also possess an α^{LELY} allele. If both mutations now occur in the same α -chain (that is to say in *cis*), which will be largely rejected by the membrane-bound β -chain, it will be the normal α -chain that will be predominantly incorporated into the network. In this case the polymorphism acts to rescue the double-heterozygote from the worst consequences of the hereditary elliptocytosis. But if the mutations occur



Consequences of double-heterozygosity on the clinical severity of hereditary elliptocytosis. *a*, In the normal membrane cytoskeletal network, the spectrin tetramers, which are made up of two $\alpha\beta$ dimers, associated head-to-head, extend between the lattice junctions (containing short actin filaments and other proteins). *b*, In severe hereditary elliptocytosis, mutant spectrin molecules are defective in respect of this kind of association, and the network is interrupted. *c*, The tetramer is generated by the complementation of two incomplete triple-helical repeating units, one from the α -chain, the other from the β -chain. The arrow indicates the region in which the disruptive amino-acid replacement most commonly occurs. *d*, The process of assembly: the β -chain is synthesized first and attaches to its site (ankyrin) on the membrane. It then takes up α -chains, which appear in large excess in the cytosol. Lateral association between the α - and β -chains begins at a nucleation site, comprising four strongly interacting pairs of repeating units at the ends of the two chains; the more weakly interacting units can then pair off along the chains as shown. In an individual heterozygous for the α^{LELY} polymorphism, in which the nucleation site is impaired, the β -chain will take up the wild-type α -chain preferentially (probably more for kinetic than thermodynamic reasons). If the self-association defect is in the same allele as the α^{LELY} polymorphism, the deleterious spectrin will be rejected, whereas if it is in the wild-type allele it will be preferentially carried onto the membrane and the situation depicted at bottom left will result.

in different α -chain alleles, then the normal β -chain binding site will carry the defective dimer binding sites onto the membrane, and the proportion of functional spectrin tetramers will be greatly suppressed; the malign effects of the elliptocytic mutation will thus be disastrously amplified.

It may be small consolation to the unfortunate patients that molecular genetics and protein chemistry have so harmoniously combined to explain the basis of their disease. There are, however, altogether milder forms of hereditary elliptocytosis, caused probably by amino-acid replacements that only weaken, rather than annihilate, the association of

spectrin dimers. Even in such a case⁶, an offspring with an elliptocytic α -chain mutation of this kind, together with α^{LELY} in *trans*, is more noticeably affected than the father with no α^{LELY} allele. □

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CLIMATE CHANGE

Fast flickers in the tropics

Rainer Zahn

EVIDENCE for fast and abrupt climate variability which is documented in high-latitude environmental records from the subpolar North Atlantic¹ and the Greenland ice sheet^{2,3} has shaken the palaeoclimate community over the past two years. During the last glacial period and the glacial-to-interglacial transition, high-amplitude climate anomalies with mean temperature changes of up to 7 °C occurred, lasting for several hundred years and then disappearing within decades. By about 9,000 years ago, the climate had steadied. But what was happening meanwhile at lower latitudes? Writing in *Earth and Planetary Science Letters*, Gasse and van Campo⁴ conclude that climate in tropical regions did not always march in step with that at higher latitudes.

In the Arctic–subarctic region, momentum for the rapid climate oscillations was provided by the glacial and deglacial environmental boundary conditions: periodic instabilities of the large ice sheets surrounding the North Atlantic during the last glacial period and their later collapse in the course of deglacial warming provided enough energy to result in fast high-amplitude climate anomalies. Once the North American and European ice sheets had reached their final states of disintegration, about 9,000 years ago, environmental conditions around the high-latitude North Atlantic became stable.

This may not be true for tropical and subtropical climates. A large body of continental palaeo-data from Asia and Africa exists, all of which points to abrupt post-glacial climate fluctuations in the tropics and subtropics. Reviewing these data and compiling the most revealing of them, Gasse and van Campo⁴ show that even after North Atlantic climates had calmed down, the continental monsoon

domains in Asia and Africa experienced a series of abrupt short-term phases of intense aridity. They use various indicators that monitor the history of rainfall and evaporation to infer fluctuations in the hydrological balance in western Tibet, Rajasthan, Ethiopia, western Sahara, Sahel and subequatorial Africa (see figure). The data suggest that the post-glacial period in these areas started with an extensive humid phase which was then followed by a series of drought periods. Before this initial humid phase, an older dry event (11,000–13,000 calendar years ago) occurred that was coeval with the Younger Dryas cold spell; this was when temperatures around the North Atlantic region dropped to almost full-glacial values. Even though this coincidence suggests that a link may exist between tropical climate and high-latitude surface conditions, the mechanisms to explain this interaction are complex and are not well understood.

Monsoonal circulation determines a wide range of environmental conditions, from continental desertification and dramatic river flooding to marine biological productivity of wind-driven oceanic upwelling zones. As such, monsoonal climate has long attracted the attention of palaeoclimate researchers and climate modellers in joint efforts to determine possible forcing mechanisms, and their influence on monsoonal variability. Primary forcing factors which exert control on monsoonal variability on timescales of 10³–10⁵ years are incoming solar radiation, transport of heat from one hemisphere to the other, and high-latitude glacial and interglacial boundary conditions^{5–7}.

For instance, the models predict temperature decreases by 2–3 °C and a decrease in rainfall by over 10 per cent

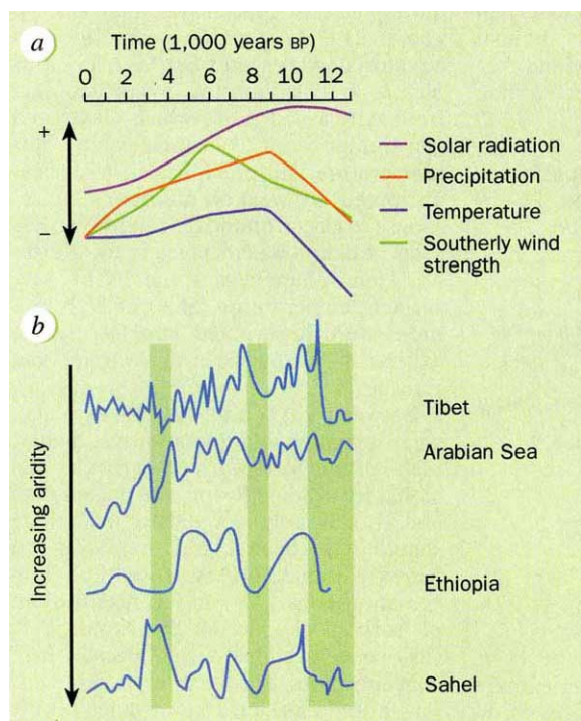
during the Last Glacial Maximum (LGM, about 21,000 calendar years ago) in equatorial Africa and South Asia⁶; this picture is corroborated by palaeo-data from Africa and Asia, which Gasse and van Campo refer to in their paper. This temperature and precipitation pattern is attributed to weaker monsoons in response to glacial boundary conditions: less solar radiation was received in the Northern Hemisphere during the LGM, sea-surface temperatures (SST) at high latitudes were lower, and large ice sheets existed in northern North America and Europe.

Between 9,000 and 11,000 years ago, when incoming solar radiation was higher, high-latitude SSTs warmer than today and global ice volume lower, the palaeo-data and model output indicate that there should have been higher humidity and increased temperatures caused by stronger monsoons. Varying concentrations of carbon dioxide in the atmosphere (low concentrations during glacials, high concentrations during interglacials) and low-latitude glaciers, for instance on the Tibetan Plateau, are also important factors affecting the strength of monsoonal circulation.

In view of the large-scale environmental changes that were associated with climate shifts from full-glacial to full-interglacial conditions, the response of monsoonal circulation — like that of any other component of Earth's climate system — is not too surprising. But the post-glacial short-term anomalies that are documented in tropical and subtropical records of climate fluctuation really do push our understanding of the climate system to its limits. These anomalies occur during a state of apparent stability of external boundary conditions, which change only little. Yet the existence of these anomalies shows that tropical climate is very sensitive to subtle changes of its forcing mechanisms.

Variations in cross-equatorial heat transport by ocean surface currents in the thermohaline 'conveyor-belt' circulation may well have contributed to the short-term instabilities in the monsoonal climate domains^{8,9}. Comparison of the rainfall pattern in the Sahel region with the global ocean SST pattern shows that, during the

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Climate change and droughts throughout tropical-subtropical Asia and Africa during the past 13,000 years. *a*, The variation of solar insolation¹¹ and environmental parameters; temperature, precipitation and southerly wind strength are inferred from a numerical climate model⁶. *b*, Four different environmental records, each indicating relative aridity: for Tibet, a pollen record from Lake Sumxi in the western Tibetan highlands, indicating changes in vegetation⁴; Arabian Sea, variations in carbonate concentration along a sediment core drilled off the coast of Oman (decreasing carbonate concentrations after 5,500 years BP indicate increased deposition of airborne dust because of enhanced aridity in Arabia¹²); Ethiopia, water level variation in Lake Abaya in the Ethiopian rift⁴; Sahel, an oxygen isotope record of a former lake in the Chad basin⁴ (variations in isotope composition of the ancient lake's water were driven by the balance between precipitation and evaporation). In each case, aridity increases downwards. Major dry spells occurred at 11,000–13,000, 8,000–9,000 and 3,000–4,000 years ago (all ages are given here in calendar years). The data also show differences in climate trend as one moves from the Indian monsoon domain to that of Atlantic Africa. High-latitude forcing of climates in Atlantic Africa may account for the differences; changes in heat transport to the north in the Atlantic sector of the global thermohaline 'conveyor-belt' presumably affects the world-wide pattern of inter-hemispheric temperature contrast which determines the moisture flux to tropical Africa¹⁰.

past 80 years, dry episodes including the current drought in the Sahel have been correlated with warming of the surface ocean in the Southern Hemisphere and the North Indian Ocean, and cooling of the North Atlantic and the North Pacific¹⁰. The net effect of this world-wide SST anomaly pattern was a reduction of moisture flux into the Sahel zone. Such changes may help to explain the climate pattern of a restricted geographical region, although they may not uniquely explain the pattern of Holocene climate throughout tropical and subtropical Africa and Asia.

Two steps seem to be essential if we are

to gain better knowledge about coupled ocean and atmospheric forcing of continental climate at all latitudes. First, atmospheric general circulation models ought to be coupled to a dynamic ocean thermohaline circulation which changes in accord with changing external boundary conditions (and ideally also with changing internal boundary conditions which follow from the external changes). Models do not properly account for this at present; the NASA GISS model, for instance, which was used to test monsoonal sensitivity to external boundary conditions⁷, prescribes ocean heat fluxes in a series of 'correction fluxes' which vary as a function of latitude so as to balance the offset between purely insolation-driven and observed atmospheric temperature gradients. That is, the real ocean's dynamics are excluded from the sensitivity test, thus limiting the output to the atmosphere's dynamics.

Second, hard palaeo-data are needed to test and refine the models. Ideally, the data would be acquired from marine sediment cores taken along the flow path of the upper limb of the ocean's conveyor belt. Critical areas to be studied are the Pacific-Indian heat-flow pathway through the Indonesian archipelago, the Agulhas current and associated flow of surface water and heat around South Africa into the South Atlantic, the North Atlantic and North Pacific convection and return-flow regim-

es, and the complex equatorial current systems. Research programmes such as MESH (Marine Aspects of Earth System History) and IMAGES (International Marine Global Change Study) are just appearing on the horizon of global change studies to guide palaeo-oceanography and palaeoclimate research into the next century. As such, they should provide the international and multi-disciplinary forum needed to carry out such research. □

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DAEDALUS

Scan the Heavens

Now that the heroic succession of interplanetary space probes is beginning to falter, Daedalus is thinking of other ways to study our planetary neighbours. He points out that, like the solar wind, a particle beam could travel through space almost indefinitely. Electron beams can be generated with peak energies of many gigawatts; during the Star Wars project they were seriously considered as space weapons. So Daedalus is inventing the scanning electron telescope.

His idea is to put a beam-launching satellite into some low orbit, and aim intense particle beams at the planets. The obvious problem is charge-neutrality. A satellite which launched a single strong electron beam would accumulate such a vast positive charge that it would soon attract the beam disastrously back onto itself. It would have to launch an electron beam and a positive-ion beam at the same time, and ideally at the same target. The two parallel beams would then attract each other. In the course of their journey, they might entwine to form, in effect, a stable and intensely hot plasma jet, which would not spread out however far it travelled. The beam would be given a known, time-coded modulation as an identity marker.

Aiming and steering such a beam would pose tricky problems. How to sweep it accurately across the face of a planet or asteroid from many millions of kilometres away? Once the beam was locked on a path intersecting that of the target, small variations of its energy might slightly alter its trajectory through the interplanetary field, giving the required scan. At its destination, the beam would induce X-ray and visible luminescence at the point of impact, and release backscattered and secondary particles from it. The luminescence would be easier to detect. Since it would be code-modulated at the beam frequency, any crude earthbound telescope trained in its general direction could pick it up. Background illumination from the target, the atmosphere and so on, would be unmodulated and easily rejected. The round-trip delay revealed by the modulation would give information on the fields traversed by the beam. Aimed at an asteroid with no atmosphere, a metre-wide beam could resolve features of its surface on the same scale, and reveal their composition from their X-ray fluorescence. Aimed at a planet, the beam should induce strong atmospheric aurorae, illuminating its meteorology. Aimed at the Moon, it could write luminous advertising slogans visible from Earth, to pay for the project.

David Jones

Avian endemism and forest loss

SIR — New statistics on tropical deforestation make grim reading¹. Overall annual losses of open and closed forests averaged 15.4 million hectares during 1981–90 (equivalent to 0.8% per annum)¹, and appear to be accelerating^{2–4}. Here we show that these results reveal an additional cause for concern. By integrating the deforestation data with an extensive analysis of patterns of avian diversity⁵, we provide quantitative evidence that forest clearance is most pronounced in areas of high biological uniqueness. Tropical deforestation may thus result in far greater loss of species than the average global figures suggest.

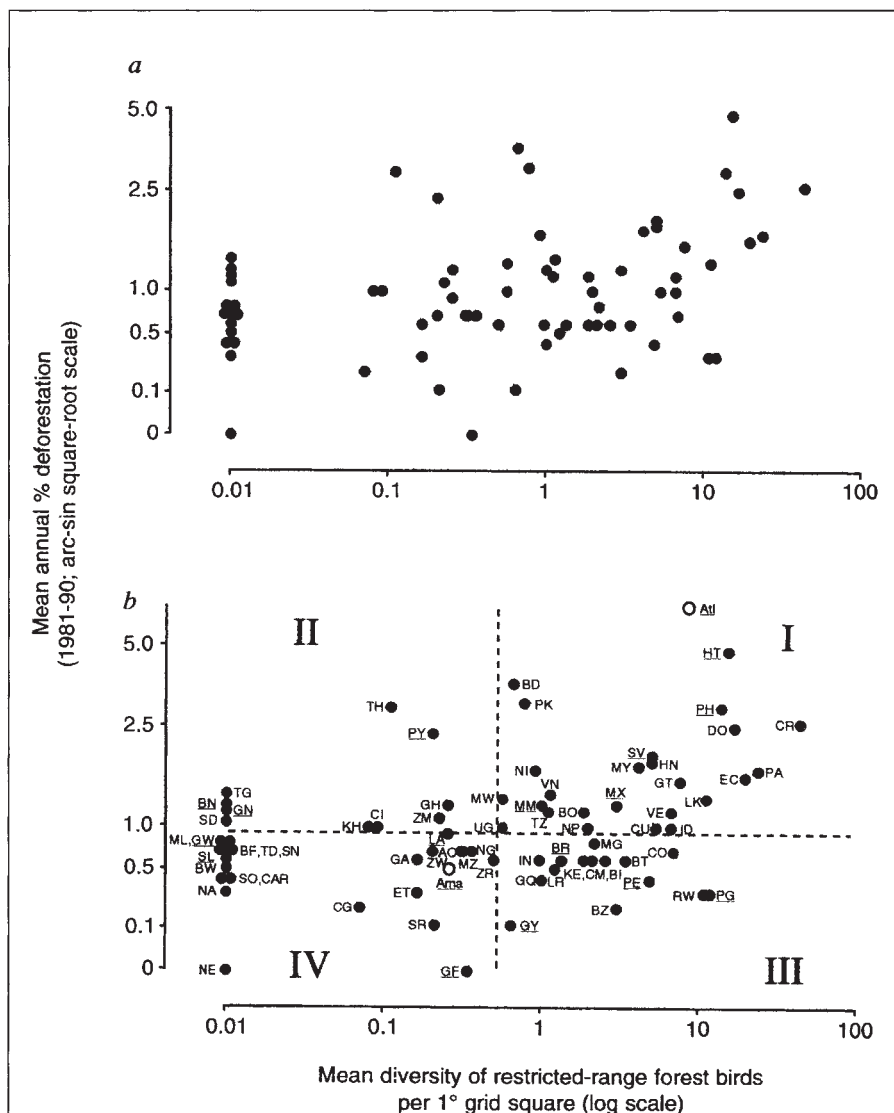
The bird data focus on a relatively well-known yet highly vulnerable indicator group: those 2,609 species with breeding ranges below 50,000 km². This set includes nearly 80% of all threatened birds, and exhibits considerable spatial congruence with endemic and threatened species in a wide array of other animal and plant groups^{5–7}. We calculated the number of restricted-range forest birds recorded in each 1° square which originally supported tropical forest (as indicated by potential vegetation maps). Mean scores across all of a country's squares then provided an area-independent measure of the biological value of each nation's forest estate, to compare with current levels of deforestation.

Our results provide the first systematic evidence of a widespread positive association between the rate at which tropical forests are being cleared and their biological importance (*a* in the figure; Pearson's $r = 0.35$, $N=72$ countries, $P<0.01$). On average, countries with large numbers of restricted-range forest birds per unit area are losing their forests faster than countries with low levels of endemism. Mechanisms underlying this disturbing relationship remain unclear. One possibility is that long-term isolation of forests on real or habitat islands surrounded by

land suitable for human settlement facilitates deforestation as well as allopatric speciation⁸. Both processes may be less likely where forests instead form large blocks of continuous habitat.

The observed correlation between

deforestation and avian endemism underlines the need to incorporate data on threat as well as biological value when assessing conservation priorities. We identified where additional forest conservation might be most urgently needed by separating countries into quadrats defined by median values for deforestation and avian endemism (*b* in the figure). Several coun-



a, Relationship between mean annual % deforestation of tropical countries (compound rate, 1981–90) and the mean number of restricted-range forest birds recorded in 1° grid squares of forest. Note both variables are transformed and are independent of the confounding effects of variation in forest area. To include countries with no restricted-range species in analyses of logged data, 0.01 was added to the score of every country. Broad definitions of forest (including open woodland as well as more closed formations) were used in calculating both variables^{1,5}. Only grid squares covered mostly by land and for which potential vegetation maps showed most of this was originally forest were included; this explains the omission of several island states. *b*, Priority ratings for forest conservation based on median scores for deforestation and avian endemism. Countries are labelled using ISO codes. Those known to have <5% of their remaining moist forest in protected areas are underlined, though the paucity of data means this list is incomplete. Sector I countries (with high deforestation and endemism) merit highest priority; sector II (high forest loss, low endemism) includes countries where urgent intervention may be less rewarding in terms of conserving endemics; sector III countries (low deforestation, high endemism) are long-term priorities for forest conservation, and may be cheaper to protect if dealt with now rather than later; countries in sector IV (low deforestation and endemism) may be relatively low priorities for immediate action. The unreliability of priority ratings for large, heterogeneous countries is illustrated by Brazil — overall this lies in sector III, but if analysed separately, its two main blocks of moist forest (open circles) lie in sectors I (Atlantic forest, Atl) and IV (the Legal Amazon, Ama).

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IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Burning forest, Tanimbar, Indonesia

tries in the top priority quadrat (sector I — high endemism and high deforestation) include threatened hotspots identified by earlier analyses^{9–11}, but our approach also highlights additional priority nations in central America and the Caribbean. In particular, we propose greatest importance should be given to countries like the Philippines and Haiti, which combine pronounced endemism and deforestation with very poorly developed networks of protected areas.

We stress that the present results are

in revealing a worrying link between forest loss and biological value, and in helping to construct an agenda for remedial conservation action.

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subject to several significant caveats. Key sites for one group of species do not always match those for other taxa¹². Moreover, using national averages for deforestation and endemism may yield misleading priority ratings where large, heterogeneous countries contain smaller hotspots facing acute threats (see *b* in the figure for the example of Brazil). Our analyses also ignore the effects of forest fragmentation, which generally increases with overall loss¹. Nevertheless, we believe our findings are of broad significance, both

and TMV RNA, and from poly(A), is proportional to the concentration of the enzyme. No bases are liberated from poly(C), poly(G) and poly(U) (as assayed spectrophotometrically, results not shown).

Thus our results demonstrate that the action of at least some saporins is not limited to rRNA, but is exerted on all tested ribo- and deoxyribopolynucleotides. Obviously, these RIPs do not release adenine only from the GAGA minimal sequence⁵, and actually seem not to have specificity for any nucleotide sequence, although they do not act on adenosine or ATP. The effect of these proteins on globin messenger RNA and on transfer RNA suggests that they may inhibit protein synthesis by altering not only ribosomes, but also mRNA and tRNAs. These proteins would be more appropriately called polynucleotide: adenosine nucleosidases and the question arising from this is what is their natural substrate?

It has previously been supposed that RIPs can more easily enter cells damaged by viral infection, thus inactivating ribosomes, killing the infected cells, and arresting infection^{6–9}. But our results show that saporins, at least, acting on substrates other than rRNA, could directly inhibit the replication of viruses by damaging their genomic or messenger RNA. Because these saporins depurinate TMV genomic RNA at concentrations similar to those present in plants, it is possible that this is a mechanism whereby these RIPs exert their antiviral activity in nature. It remains to be seen whether this is the only function of RIPs, or whether they have a role in normal plant metabolism.

Because of their effect on all types of RNA and DNA, these proteins would kill any cell, including bacteria. Thus in plant cells these RIPs would presumably either be segregated within the cytoplasm, or exist in an inactive form, or be kept inactive by an inhibitor.

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Unexpected activity of saporins

SIR — A group of plant proteins with antiviral activity inhibits protein synthesis by irreversibly damaging ribosomes, and hence are provisionally called ribosome-inactivating proteins (RIPs, reviewed in ref. 1). These proteins can be divided into type 1 RIPs, single-chain proteins and type 2 RIPs (ricin and related toxins), consisting of an active A chain bound to a B chain with the properties of a galactose-

specific lectin. Type 1 RIPs, as well as the A chain of type 2 RIP, are *N*-glycosidases which depurinate ribosomal RNA by cleaving the *N*-glycosidic bond of a single adenine in a specific position of rRNA (*A*₄₃₂₄ of rat liver 28S rRNA)², although some RIPs cleave more than one adenine from rat liver rRNA³. We report here that saporin-L1, a RIP from the leaves of *Saponaria officinalis*⁴, also releases adenine residues from various RNAs, from DNA and from poly(A), but not from ATP, adenosine or dATP.

When assayed with bacteriophage MS 2 genomic RNA as substrate, 0.2 μ M saporin-L1 released more than 130 mol adenine per mol MS 2 RNA. Saporin-L2 and saporin-R2 are half as active, whereas all other RIPs assayed (49 type 1 and the A chains of 4 type 2 RIPs) are inactive or release only traces of adenine from MS 2 RNA.

Saporin-L1 releases several hundred pmol adenine from various other RNAs, poly(A) and DNA (see table). The amount of adenine released from MS 2

EFFECT OF SAPORIN L-1 ON VARIOUS ADENINE CONTAINING SUBSTRATES

Substrate	Adenine released (pmol)
Poly(A)	2,038
Globin mRNA (rabbit reticulocytes)	1,587
DNA (herring sperm)	747
<i>Bryonia dioica</i> poly (A) — RNA	524
<i>Escherichia coli</i> rRNA (16S + 23S)	435
<i>Saccharomyces cerevisiae</i> tRNA ^{Phe}	425
Tobacco mosaic virus (TMV) genomic RNA	371
Bacteriophage MS 2 genomic RNA	336

Reaction mixtures were contained in a final volume of 50 ml 20 mM Tris-HCl buffer, pH 7.8, 100 mM ammonium chloride, 10 mM magnesium acetate, 0.07 μ M saporin-L1 and substrate (10 μ g RNA or poly (A), 12 μ g DNA). Incubation was at 25 °C for 40 min. Adenine released was determined as in ref. 3. Ricin A chain under the present experimental conditions has no effect on *E. coli* rRNA, in agreement with Endo², who showed the release of one adenine residue per mol of each species of RNA (equivalent to 11.4 pmol in our experimental conditions) at much higher reactant concentrations than we used in our experimental system.

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Climate, CO₂ and plant abundance

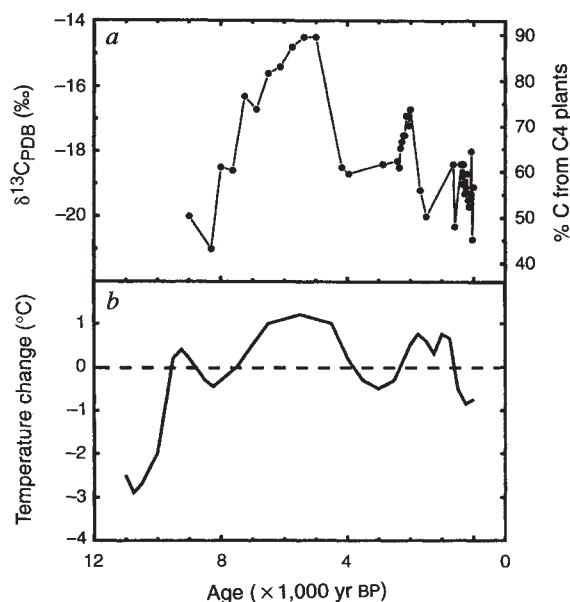
SIR — Cole and Monger¹ presented $\delta^{13}\text{C}_{\text{PDB}}$ and $\delta^{18}\text{O}_{\text{PDB}}$ records for pedogenic carbonates in palaeosols representing the past 30,000 yr BP (before present) in the northern Chihuahuan desert, New Mexico. Their $\delta^{13}\text{C}_{\text{PDB}}$ values indicated that C4 grassland dominated from 30,000 to 8,000 yr BP, but was replaced around 8,000 yr BP by a C3-dominated flora (probably desert shrubland). They proposed that this C4-to-C3 shift was caused by an increase in atmospheric CO₂ concentration (pCO₂) from 200 to 280 p.p.m.v. during the late Quaternary. They also recommended that $\delta^{13}\text{C}_{\text{PDB}}$ values of palaeosols could be used as a proxy for past pCO₂ changes. But the ecophysiological characteristics of C3 and C4 plants, uncertainty regarding ecosystem-level responses to pCO₂ increase, and $\delta^{13}\text{C}$ measurements of palaeosols in other regions, indicate that these results should be interpreted with caution.

A change in atmospheric pCO₂ from 200 to 280 p.p.m.v. significantly increases photosynthesis, water-use efficiency and biomass production of C3 species at the leaf and whole-plant levels². However, even at the present pCO₂ of 350 p.p.m.v., C4 species still have quantum yields, photosynthetic rates and water-use efficiencies comparable to or greater than those of coexisting C3 plants^{3,4}. Some C4 species also show enhanced carbon and water relations and increased growth with increasing pCO₂ (refs 3,5). In addition, net photosynthesis by C3 plants is reduced 25–40% by photorespiration under present atmospheric CO₂ and O₂ concentrations⁴; this reduction may have been greater during the late Quaternary when the CO₂/O₂ ratio was lower than at present. Thus, although carbon and water relations of C3 plants may have improved as pCO₂ increased from 200 to 280 p.p.m.v. during the late Quaternary, ecophysiological evidence does not suggest this increase in pCO₂ would have shifted competitive interactions to promote replacement of C4 grassland by C3 desert shrubland. In fact, C4 plants still comprise 50–90% of standing crop biomass in some contemporary Chihuahuan desert plant communities⁶, and C4 plants continue to dominate many grasslands throughout the world characterized by high light intensity, warm temperatures and limited water availability⁴.

Atmospheric pCO₂ is only one of many environmental factors that can influence plant communities, and direct effects of pCO₂ on leaf and whole-plant ecophysiology may be poor predictors of community, ecosystem or regional vegetation composition and dynamics⁷. It is possible that

late Quaternary changes in pCO₂ enhanced carbon and water relations of C3 and/or C4 plants and played some part in the evolution and dynamics of plant communities with coexisting C3 and C4 species. However, interactions between pCO₂ and other environmental limitations (temperature, amount and distribution of precipitation, soil fertility, frequency and intensity of disturbance) or intrinsic biological characteristics of the plants themselves (allocation patterns, growth form, life history) could potentially reduce or negate plant responses to elevated pCO₂ (refs 5, 7).

If the C4-to-C3 shift in the Chihuahuan desert was driven by rising pCO₂, then similar shifts should be evident elsewhere at 7,000 to 9,000 yr BP. The $\delta^{13}\text{C}_{\text{PDB}}$ of organic carbon in palaeosols in the southern Great Plains⁸ (a in the figure) contradict this prediction, and indicate that the relative importance of C4 grass production increased from 9,000 to 5,000 yr BP. Estimates of C4-derived carbon at this site correlate with both mean global temperature variations (b in the figure) and regional climate reconstructions⁸ over the past 9,000 yr BP, consistent with previous research demonstrating that C4 distribution and performance are correlated strongly with temperature⁴. Other $\delta^{13}\text{C}_{\text{PDB}}$ records from palaeosols throughout the Great Plains^{9–11} also document significant increases in C4-derived carbon that correlate with climate, but not the pCO₂ record, during the early and middle Holocene. These



a, $\delta^{13}\text{C}_{\text{PDB}}$ of organic matter in palaeosols and sediments from Fort Hood Military Reservation in the southern Great Plains of central Texas. b, Global mean temperature variations during the Holocene. Stratigraphy, geomorphology, chronology and isotope methodology associated with the palaeosols have been described in detail elsewhere⁸. Precision (± 1 s.d.) of $\delta^{13}\text{C}_{\text{PDB}}$ values was ≤ 0.2 ‰. The proportion of carbon derived from C4 plants was estimated by mass balance using -13 ‰ and -27 ‰ as mean $\delta^{13}\text{C}_{\text{PDB}}$ values for C4 and C3 plants, respectively. Holocene global temperature variations (b) are adapted from ref. 13 and expressed relative to mean global temperature near the beginning of the twentieth century ($\sim 15^\circ\text{C}$).

results^{8–11} indicate that $\delta^{18}\text{C}_{\text{PDB}}$ of soil carbon is not always a reliable proxy for pCO₂.

Cole and Monger¹ report that their C4-to-C3 vegetation change at 8,000 yr BP was coincident with increased aridity in the southwestern United States, as indicated by climate modelling, palaeovegetation and geomorphic reconstructions, and packrat middens. This shift from C4-to-C3 might, therefore, reflect the replacement of C4 mesophytic grasses by C3 xeromorphic woody growth forms better adapted to warmer, drier conditions despite having the C3 pathway. However, because $\delta^{18}\text{O}_{\text{PDB}}$ in their pedogenic carbonates varied little at the time of the C4-to-C3 transition, Cole and Monger argued that climate was not responsible for the vegetation change. Considering the uncertainties associated with the climatic significance of $\delta^{18}\text{O}_{\text{PDB}}$ in continental carbonates¹², this result may not constitute unequivocal evidence for constant climate. The area studied in ref. 1 lies near the ecotone between the grassland and desert biomes of North America, where desert scrub and grassland communities intergrade over broad areas. As a result, small changes in temperature and distribution and amount of precipitation that might be undetectable in the $\delta^{18}\text{O}$ record, and/or changes in disturbance regime, could produce relatively large changes in

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vegetation composition. Although the precise mechanism responsible for the vegetation change documented by Cole and Monger remains unknown, it seems unlikely that increased pCO_2 was the only driving force.

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MONGER AND COLE REPLY — The purpose of our paper¹ was to show the correlation between a decline of C4 grasses recorded by soil $\delta^{13}C$ values and increased pCO_2 recorded by ice cores²⁻⁵. We think this correlation is important in the light of recent experimental results showing that increased pCO_2 may give C3 plants a competitive advantage⁶, and that the expansion of Miocene C4 biomass was potentially related to lower pCO_2 levels⁷.

We agree with Boutton *et al.* that a pCO_2 -driven shift did not happen in all C4 ecosystems worldwide approximately 7,000–9,000 years BP. We did not state that the C4-to-C3 isotope signal would be universal, but pointed out that the signal we observed was associated with an arid alluvial fan system¹. Such alluvial fan systems located in desert scrub/grassland ecotones may offer the best place to 'prospect' for floral responses to changing pCO_2 for two reasons. First, well-preserved palaeosols necessary for the isotope record are common. Second, these areas are ecologically fragile because their soils often have low water-holding capacities, making them susceptible to environmental stresses. In contrast, intermontane basin soils adjacent to our study area that receive runoff and have high water-holding capacities exhibit only moderate isotope shifts at 7,000–9,000 years BP⁸.

Therefore, considering the magnitude of pCO_2 change during the last deglaciation, we would not expect to see a floral shift driven by increasing pCO_2 within prairies of central Texas or elsewhere. Using the example of Boutton *et al.*, an

increase in C4 grasses occurred from 9,000 to 5,000 years BP in central Texas⁹, whereas a decline in C4 grasses occurred at the same time on alluvial fans in southern New Mexico¹. Both areas experienced aridity and increased pCO_2 . In southern New Mexico increased pCO_2 , which potentially gives C3 desert scrub a competitive advantage⁶, would have intensified the effects of aridity to promote a loss of C4 grasses. In contrast, the benefits to C3 trees and C3 grasses from increased pCO_2 in central Texas were probably offset by aridity, which promoted C4 prairie expansion.

Important clues about the relative roles of pCO_2 and climate on vegetation shifts can be provided by $\delta^{18}O$ values. We acknowledge the uncertainties associated with using these values in pedogenic carbonate as climatic indicators. But unlike previous studies^{10,11} showing parallel shifts in $\delta^{18}O$ and $\delta^{13}C$, purportedly due to climatically driven vegetation changes, our results show profound $^{13}C/^{18}O$ discordance. Because $\delta^{18}O$ values remained relatively constant ($\pm 1\text{‰}$) whereas $\delta^{13}C$ values changed approximately 6–8‰, we regard the discordant trajectories as an indication that another variable, pCO_2 , had an important influence on the vegetation change.

There is no question that a number of variables cause C4–C3 vegetation shifts. We did not claim that pCO_2 was the only driving force. However, where fragile arid and semiarid environments are well-preserved in the geological record, and other variables such as climate change are understood, we believe $\delta^{13}C$ values in palaeosol carbonate may be an important indicator of past CO_2 changes.

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CO₂ effects at high temperature

SIR — Oechel *et al.*¹ report that initial increases in carbon fixation by an Arctic tundra ecosystem exposed to elevated CO_2 concentrations (680 p.p.m.) were not detectable after 3 years at ambient temperatures; in contrast, experimental areas exposed to elevated CO_2 and temperature (+4 °C) continued as net sinks for CO_2 3 years after the experiment began, albeit at a slightly lower level than in year one. Unfortunately, Oechel *et al.* do not report results for enhanced temperatures only. The data refer to control and enhanced CO_2 at ambient temperatures and enhanced CO_2 at enhanced temperatures. Responses to enhanced temperatures alone are not reported, although the methods suggest that the measurements may have been done. On the data provided it is therefore impossible to distinguish an ecosystem-level response to enhanced temperatures alone, from the effects of enhanced temperatures in combination with enhanced CO_2 . Simply, enhanced CO_2 uptake in year three may have been a temperature effect.

We are now exploring in the Ecotron^{2,3} ecosystem-level impacts of enhanced CO_2 , enhanced temperature, and their combination in a fully factorial design, across several plant generations, allowing us to test Oechel *et al.*'s important conclusions.

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Replication step

SIR — In your article "Guesswork and randomness" (*Nature* **372**, 30; 1994), you state in regard to Spiegelman's molecular evolution experiments on Q β that "selection for the replicase giving the most rapid replication of the whole virus eventually yielded a dramatically shorter enzyme". This is not so. The experiment, which involved serial replication of Q β RNA by purified Q β replicase *in vitro*, yielded a greatly shortened (biologically inactive) RNA, in which sequences required for recognition by the replicase were conserved but most coding sequences were eliminated. The selective advantage of this RNA *in vitro* was that it was replicated faster than full-length wild-type Q β RNA. No "shorter enzyme" resulted or was involved in this experiment.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references.

Dilemmas and missions

A. G. Keller

The Fontana History of Technology. By Donald Cardwell. *Fontana*: 1994. Pp. 565. £9.99 (pbk). To be published as *The Norton History of Science in the United States* by Norton in January 1995, \$35 (hbk), \$18.95 (pbk).

The Power of the Machine: The Impact of Technology from 1700 to the Present Day. By R. A. Buchanan. Penguin: 1994. Pp. 299. £7.99 (pbk). First published by Viking in 1992.

Power from Wind: A History of Windmill Technology. By Richard L. Hills. Cambridge University Press: 1994. Pp. 324. £45, \$59.95.

WHEN Fontana decided to produce a series devoted to the history of science, a volume on technology was included. As each volume aims to examine a "particular field of science", does this imply that technology is a science, on a par with astronomy or chemistry? Surely technology is as varied and as wide ranging as science; and a comprehensive history would have to cover many volumes.

Technology is of course considered by some people to be a discipline that is dependent on the scientific search for knowledge, undeserving of the name of science and one that often distorts, even prostitutes, pure science for the sake of profit or military victory. There has indeed been a long debate on the relationship between science and technology, in which the supposed 'scientification' of modern technology is seen as the proper consummation. But science today is so dependent on instrumentation, and technological research and development so scientific in its procedures, that the old boundaries are fading away. Still, with 'technology' claiming only one book in the new series, does this mean that it has a Cinderella role in the palace of the history of science? While mathematics and cosmology reside in the hall gazing into the remotest stretches of time and space, poor Cinders down in the kitchen gets her hands grubby providing the hardware and earning the grants for basic research.

Perhaps it could be put another way. Science really means knowledge — no more and no less than what we feel confident we know about any given subject. As such, 'science' is neutral. But if a certain recent extension of our knowledge is reliable, it can be turned to our advantage in the form of a new instrument, a new material or a new process. To be sure, the advantage to one person may not be the advantage to all. The development of atomic weapons from the discovery of nuclear fission might be a case in point.

These thoughts are inspired by the appearance of two major surveys of the whole history of technology, the first attempts at such all-embracing histories for many years, and written as summa-

tions of their work by the two leaders of the history of technology in the United Kingdom. Cardwell's *Fontana History* follows Buchanan's *Power of the Machine*, originally published two years ago and now out in paperback. First the authors must decide whether technology includes

eleventh century, and to a restricted area of northwestern Europe, then clearly this was a revolutionary invention, and one that spread quite rapidly across much of northern Europe within a century. Thereafter development was much slower, and evolutionary, up until very recent research on wind-engines, designed to employ the wind to generate electricity. In the end Hills has to admit that the wind is so variable in velocity and direction that it can never make up much of our energy budget, like other sources of power too "dilute" in Cardwell's term to be the answer to our problems.

The distinction between "revolutionary" and "evolutionary" innovation is also Cardwell's; he compares this view with T. S. Kuhn's "revolutionary" and "normal science". As Cardwell observes, the revolutionary developments are often launched by outsiders who have not been

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

A windmill as depicted in an early fourteenth-century manuscript in the Bodleian Library.

all the tools and methods by which humans gain a living from the environment — which might involve the earliest stone implements as well as manual implements still in common use such as garden forks, violins, toothbrushes — or whether the term should be restricted to 'scientified' procedures? Cardwell distinguishes between technics and technology: "we have on the one hand crafts, technics and invention; on the other hand technology, applied science and invention". Like Buchanan, he deals with both, and describes "the secular progress of technics" through innovations that were not founded on "systematic knowledge", before we reach the age of "science-and-technology".

The exploitation of wind power might be a good illustration of revolutionary technics. Hills explains that he will deal with the changing techniques used to exploit the wind's energy more efficiently. If the first post-mills can be pinned down to a few decades in the middle of the

schooled in current engineering practice and are not personally involved in the technologies they are going to alter so radically, or even replace. Buchanan too sees all these numerous changes in different industries forming a grand Technological Revolution, which begins "slowly but steadily since the end of the Middle Ages" (if not earlier) and gathers strength from the increased use of wind and water power, followed by the introduction of steam power and advances in ferrous metallurgy, notably in the manufacture of cast iron and steel that have ushered us into a "New Iron Age" since the nineteenth century. Basically, Cardwell seems to be thinking on similar lines. Both look for explanation for this onward movement in the sociopsychology of Western Europe in late mediaeval and early modern times. Buchanan speaks of the "technological ratchet" by which one invention demands another, and so a series that may replace the technology for which the invention was originally designed; and the 'package',

the favourable environment, social and economic, that both he and Cardwell see in personal mobility, varied economies, liberal attitudes and the readiness to welcome novelty rather than closed societies and closed minds. Cardwell indeed argues that pre-Reformation Christianity favoured the development of innovative craft skills, and helped to overcome what he calls "the taboo of the natural", a fear of going against nature and natural boundaries, which "the navigators, the anatomists, and the miners" had to confront.

Cardwell and Buchanan both stand in the liberal optimistic tradition; they view the progress of technology as a rational enterprise, and clearly see what they recount as a success story. Perhaps it could be said that both tell a somewhat traditional story, concentrating on the physical technologies of the classical industrial revolution, as Cardwell acknowledges, although he and Buchanan do include medical, agricultural and chemical innovation too. As might be expected of Cardwell, who has written on the history of thermodynamics and steam engines, the power-and-energy story and the development of mechanisms predominates: he recalls Liebig's phrase about "the economy of power". Buchanan is more comprehensive. But evidently, of two books of broadly similar format, Cardwell's, being twice as long, can be more detailed, and includes diagrammatic explanations of major inventions.

Both authors remain hopeful that technological innovation has been and will remain beneficial overall. Buchanan does recognize a "technological dilemma", in as much as we are ever more dependent on our technology, but therefore more vulnerable to its abuse, and to the threat of pollution and exhaustion of resources. He feels we need to think ahead; to control these developments with some long-range thinking. We need a "technological mission", which he hopes to see in the conquest of hunger and disease on Earth, and in the "way to the stars", the exploration of space. Both will "ensure the survival of the species through greater self-knowledge, acquired through a more profound insight into the nature of the universe". Let us hope so. Melvin Kranzberg has reminded us, however, that technology is not good, bad or even indifferent. Technology is our creation and it is we who are good, bad and most often indifferent. Perhaps we can with our future development and application of technology justify the optimism of these historians. The record of the past 80 years does raise some niggling doubts, but the potential is in our hands. □

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Making waves

Willem Hackmann

The Creation of Scientific Effects: Heinrich Hertz and Electric Waves. By Jed Z. Buchwald. *University of Chicago Press*: 1994. \$75, £59.95 (hbk); £26.25, \$32.95 (pbk).

NEXT year the centenary of the radio will be celebrated by a conference at the Institution of Electrical Engineers in London, and by events in Bologna (Guglielmo Marconi's birthplace) and elsewhere. It was in 1895 that this "ardent amateur of electricity", as Marconi styled himself, and Alexander Popov constructed 'wireless' equipment using a



Heinrich Hertz (1886).

coherer detector. Marconi's apparatus received man-made signals, whereas Popov's recorded atmospheric effects and was used as a storm detector.

To this day, both Italy and Russia have laid claim to the invention of the radio. On the device's fiftieth anniversary in 1945, the Soviet government declared to a packed assembly in the Bolshoi Theatre in Moscow that 7 May was to become an annual holiday called 'Radio Day', in celebration of the occasion in 1895 when Popov's receiver was shown to the Russian Physico-Chemical Society. But pinpointing the exact date of the anniversary of such a complex piece of apparatus as the radio is notoriously difficult; most inventions have evolved over time, based as they often are on several important discoveries. Sir Oliver Lodge, for example, also became interested in electromagnetic radiation and conducted similar pioneering experiments to those of Marconi and Popov but with shorter radio waves, work for which he was knighted in 1902 (see P. Rowlands and J. P. Wilson's *Oliver Lodge and the Invention of Radio*, PD Publications, Liverpool, 1994).

It is not in fact too fanciful to trace the true birthday of the radio back to the publication in 1873 of James Clerk Maxwell's *Treatise on Electricity and Magnetism*, which with hindsight seems to have contained the germ of the idea of electromagnetic-wave radiation, or to the epochal experiments of Heinrich Rudolf Hertz at Karlsruhe in 1887. Hertz did not concern himself with the practical implications of his discovery of electromagnetic waves, despite his engineering background; this was left to others in the 1890s, such as Lodge, Popov and Marconi.

A great deal has been written about Hertz. But this book, which concentrates on Hertz's wide-ranging experimental work, is the first detailed scientific account of the extraordinary and serendipitous events leading up to his

discovery of electromagnetic waves in air — a phenomenon that turned out to be inherent in his theory but was by no means immediately obvious to him. His odyssey began with his attempt to solve the key problem in nineteenth-century physics: the question of whether an intervening medium is necessary for forces to act at a distance. His patron, Hermann von Helmholtz, in line with most European physicists, favoured the view that it was, whereas the English school thought otherwise. Maxwell had demonstrated in his classical treatise that all known magnetic and

electric phenomena could be interpreted in terms of stresses and motions of a material medium, but he gave no theory of oscillatory circuits or of the connection between currents and electromagnetic waves.

The story of what led Hertz to focus on oscillatory discharges has been told before. We learn here of his attempts in the 1870s to create order in the field of electrodynamics by casting the contending theories of Weber, Neumann and Maxwell in a form that would expose their experimentally detectable differences; of how he won a Berlin Academy prize in 1878 by solving a problem posed by Helmholtz (the determination of an implication of Weber's theory of the behaviour of electric mass), thereby establishing his reputation as a meticulous experimenter; of how Helmholtz then set a second Berlin prize problem to evaluate contending electrodynamic theories — whether changes in dielectric polarization yield electromagnetic effects (as assumed by Maxwell) or, conversely, whether electromagnetic phenomena can produce dielectric polarization; of how Hertz this time declined to

pick up the gauntlet because of the difficulty of obtaining the minute effects he would be seeking with the laboratory apparatus at his disposal; of how in 1884 at Kiel he carried out a detailed theoretical study of Maxwell's theory in an attempt to make it compatible with Helmholtz's electrodynamics, but was left frustrated because he was unable to accept Maxwell's physical interpretation of his equations, in particular his denial of action-at-a-distance; and of how in 1886 Hertz's first experiments were to determine the influence of dielectrics such as pith and paraffin on the inductive communication of sparks between primary oscillatory and detector currents.

The book captures the excitement leading up to Hertz's announcement in December 1887 that he had produced electromagnetic waves. The centrepiece is an account of how his return to the second Helmholtz problem in 1886 resulted in his discovery of this phenomenon. Hertz's every step is meticulously recorded, the aim being to uncover what his experiments actually meant to him at the time. The author recounts how Hertz's Knockenhauer spiral (used to demonstrate electromagnetic induction) was transformed into a linear device; how the discharge circuit became a spark-switched oscillator (the generator) and how the Riess spark-gap electrometer first became the detached Nebenkreis circuit and then developed into a resonator (the detector). Thus, as Buchwald points out, Hertz's route was not predetermined either by a set of specific initial goals or by the intrinsic properties of his apparatus. Each step depended on its immediate context of localized problems linked to specific bits of equipment. This allowed him to produce his resonator, a tool for investigating oscillations. It was when he turned his resonator into an electric probe for mapping the electric force near his oscillator that he came across a totally unexpected phenomenon that could be interpreted only in terms of electromagnetic waves.

Buchwald takes readers on a long and at times disorienting ride through unfamiliar territory, as he is the first to admit. But this is precisely the book's strength: it forces us to think as Helmholtz and Hertz did. Only in this way can we appreciate Hertz's achievements. A series of succinct appendices assist the reader with the more difficult contemporary concepts. We are promised a sequel dealing with the acceptance of Hertz's novel device by members of the physics community and its significance to them, a volume that I for one await with keen anticipation. □

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The problems of little and large

H. C. Bennet-Clark

Diatoms to Dinosaurs: The Size and Scale of Living Things. By Christopher McGowan. Island: 1994. Pp. 288. \$24.95.

LIVING organisms range in size from a diameter of about one hundredth that of a typical human cell to a length of more than 30 metres, with weights ranging from the infinitesimal to that of a medium-sized airliner.

The smallest organisms live at an incredible pace. The energy production of a kilogram of bacteria may reach 20 million times that of a kilogram of the Sun; no wonder fermentation produces such heat. Become larger and things slow down somewhat — we humans produce energy at only thousand times the rate of the Sun! The tiny also have briefer lives: a mere six weeks' life-span for a 2-mm-long fruitfly against the three-score-and-ten years of humans or elephants.

At the extremes of size of living organisms, the problems of scaling have a 'gee-whiz' element that makes their mere description exciting. More rewarding for the biologist is the pursuit of "why?"

Why should the differences be so dramatic? Why should a walking dinosaur have left a line of craters as footprints in the ground of Jurassic Park whereas a

pond skater can skip along on the surface tension of water? Why do horses change from a walk to a trot and then to a gallop as they speed up — and why can't elephants gallop? Why should a parrot be able to live ten times longer than a rat of the same body weight?

Many of these scaling problems have already been successfully addressed by biologists: *Scaling: Why is Animal Size So Important?* (Cambridge University Press, 1984) by K. Schmidt-Nielsen (which covers, with the literature then available, much of the same area) and a remarkably eclectic survey by T. A. McMahon and J. T. Bonner entitled *On Size and Life* (Scientific American Library/ W. H. Freeman, 1983) stand out as beacons of readability, lucid explanation and, particularly with the latter, the adept use of illustration to explain the point.

McGowan's material is interesting. He writes well and he has picked good examples. But throughout the book, I found myself wondering whether I was really getting any nearer to understanding "why?". This is because he has tended to duck the solid issues of data and equations. There are certainly some hard-enough facts such as the wing-span of the largest pterosaurs or of the smallest flying wasps; but in avoiding rigour in his treatment of the aerodynamic problems of flapping flight, as well of those of how the aerodynamics of the hang-glider-sized giants differ from those of the dust-sized tiniest insects, he fails to provide a clear insight into the reason why the contrasts are so marked. Presumably the soft



THE Nanisivik mine on Baffin Island, Northwest Territories, Canada, is famous for the unusual shape of its pyrite crystals, inherited from the flat, star-shaped twinned crystals of marcasite over which they grew. Taken from *Minerals* by George W. Robinson, which contains more than 140 photographs of the mineral specimens from the collection of the Canadian Museum of Nature. Simon and Schuster, \$40.

approach he follows throughout attempts to avoid baffling or terrifying the reader, but I merely found parts of the text irritating, confusing and even patronizing. The text is not helped by the data that do appear; some of the graphs have axes that are labelled with such vague terms as "brain mass" and "body mass" but on which neither the units nor the values are given, and confusion is twice confounded by a legend that reads "data are logarithmic". Nor is the book helped by the line drawings, which are not very life-like and contain some errors: it is the fourth finger of a pterosaur that holds out the wing-tip, not the fifth; and the aerofoil section of a crane-fly wing is corrugated and not the smooth cambered surface that is drawn.

So what are the book's merits? McGowan is at his best with dinosaurs and large tetrapod vertebrates; the chapter "Giants — Modern and Ancient" is readable, interesting and informative on the skeletal design and physiological problems of very large land animals; and his comparisons of large swimming animals from sharks through swordfish to ichthyosaurs are intriguing. And, to be fair, there was no part of the book that I did not enjoy reading — irritations notwithstanding — even though I was left wondering who the intended readership is. This is the sort of book one can give to students to fire up their interest. With its good bibliography, it should lead them into the field before they get down to the nitty-gritty of the implications of scaling. □

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Ins and outs

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Handbook of Membrane Channels. Edited by Camillo Peracchia. Academic Press: 1994. Pp. 591. \$120, £92.

An Introduction to Membrane Transport and Bioelectricity. By John H. Byrne and Stanley G. Schultz. Raven: 1994. Pp. 198. \$79.50 (hbk), \$44 (pbk).

ION channels are pore-forming proteins that regulate the permeability of cell membranes, including those of intracellular organelles such as endoplasmic reticulum and mitochondria. Channels are venerable in evolutionary terms, occurring in much the same form throughout the animal kingdom; indeed our own potassium-channel subunits will form multimers with those of fruitflies. Some channels are gated by responding to changes in the membrane electric field (more than 100,000 volts per centimetre);

others are gated by the binding of ligands, such as neurotransmitters or intracellular substances such as Ca^{2+} , ATP or inositol trisphosphate. Channels allow ionic transfer at rates in excess of 106 ions per second, faster than the turnover number of the fastest enzymes and fast enough to permit the measurement of ion flow through isolated single channels.

The achievement of Neher and Sakmann in making this isolation electrically with patch clamps, coupled with the use of molecular biological techniques to clone, mutate and express ion channels, has led to an explosion of activity. Ten years ago at a meeting of the Society for Neuroscience, an audience of some fifty out of the several thousand attending heard Y. N. Jan give an account of the molecular genetics of the Shaker mutant of the fruitfly, a mutant that lacks one type of potassium channel from its muscle, making it quiver under ether anaesthesia. Although the content of this lecture was at the time difficult for this physiologist to grasp fully, it was obvious that the findings were going to be very important. The same year, Numa's group had sequenced the sodium channels that generate nervous impulses and had already cloned the acetylcholine receptor channels that allow transmission of impulses from nerve to skeletal muscle. This decade of renaissance is now celebrated in the *Handbook of Membrane Channels*, a book dedicated to the memory of Numa, who helped begin it.

The handbook is wide-ranging, but cannot be said to be comprehensive. The contributions of its many authors are collected into sections, which start with potassium channels and end with the pores formed on fusion of a secretory vesicle with the cell membrane. The writers of the various chapters clearly have the remit of integrating electrophysiological and molecular information, and although many do not pull this off completely, several do. There is, perhaps inevitably, some overlap in content. Some editing might have avoided each of the four chapters on calcium channels beginning with much the same statement about the classification of these channels into L-, T-, N- and P-types. The presence in two consecutive chapters of almost identical but very brief discussions of the cystic fibrosis transmembrane conductance regulator (CFTR) should have been avoided. Indeed, the area surely deserved a chapter of its own. CFTR is of clinical importance, as the mutation (often a simple deletion of a phenylalanine) is so common and still fatal in young adulthood. But the basic science shows that the group of proteins to which CFTR belongs — the ATP-binding cassette proteins — sits on the fence between channels and carriers. Most members of this group do work, actively extruding compounds from cells. Curiously, as Rainer Greger states in his chapter,

the Cl^- channel formed by CFTR may require ATP hydrolysis to allow downhill movement of Cl^- .

Many monographs concerning membrane physiology, aimed at an undergraduate audience, also discuss ion channels while excluding other membrane transport processes. For this reason, the second edition of the book by Byrne and Schultz is to be welcomed. The text is written with clarity and accuracy and is highly readable. The authors' enthusiasm for historical reference is perhaps less well judged; the average 18-year-old biologist is not in my experience turned on by the thoughts of Pliny the Elder. And there are one or two areas of awkwardness. For example, the units for membrane current are introduced as coulombs per cm^2 per hr; the circuitry for studying sodium channels using patch-clamp techniques is incorrectly complex; and part of the discussion of the Hodgkin-Huxley equations seems curious. Molecular properties of transporters and voltage-gated channels are touched on; most undergraduate courses may now have integrated this aspect further into the physiology. The best chapters in the *Handbook of Membrane Channels* show us that all now should. □

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New in paperback

The New Catastrophism: The Importance of the Rare Event in Geological History by Derek Ager. Cambridge University Press, £13.95, \$24.95. "His work shares with its predecessor the qualities of originality, readability and seminality. I thoroughly recommend it" (Gordon L. Herries Davies, *Nature* **365**, 115; 1993).

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Protein superfamilies and domain superfolds

Christine A. Orengo, David T. Jones & Janet M. Thornton

As the protein sequence and structure databases expand rapidly a better understanding of the relationships between proteins is required. A classification is considered that extends the sequence-based superfamilies to include proteins with similar function and three-dimensional structures but no sequence similarity. So far there are only nine protein folds known to recur in proteins having neither sequence nor functional similarity. These folds dominate the structure database, representing more than 30 per cent of all determined structures. This observation has implications for protein-fold recognition.

ALTHOUGH the protein structure databank now contains records of nearly 3,000 protein chains, many entries are similar, and it is now well established that protein domains having more than 30% of their sequence in common adopt the same fold structures¹⁻³. Furthermore, an increasing number of proteins are being revealed that have similar folds but statistically insignificant sequence similarity. Here we examine the known protein-sequence superfamilies and structural-fold families to discover the distribution of the different fold families among known protein structures.

Several groups have described the automatic clustering of the structure databank into fold families⁴⁻⁷. Our classification embraces all of the proteins in the Protein Structure Databank in April 1993 (including pre-releases) and employs the sequence and structure comparison methods described in ref. 6. Protein sequences are compared using the standard Needleman and Wunsch⁸ algorithm, and similarity measured as percentage sequence identity. Protein domain structures are compared using the SSAP algorithm⁹. This seeks out equivalences in residue structural environments between proteins. The score returned by SSAP is normalized to lie in the range 0 to 100, irrespective of the protein sizes.

Fold definition

It is first necessary to define the criteria for identifying a new fold and for deciding when two proteins adopt the same fold. The most common definition is given by the chain topology, allowing for insertions and deletions between less related structures. These occur mainly within the loops, although sometimes complete secondary structure elements can be missing. For example, the light and heavy immunoglobulin chains contain different numbers of β -strands (7 and 9, respectively). The heavy chain has two strands inserted into the middle of the chain, which extend one of the sheets. But these structures have almost certainly diverged from a common ancestor and are usually considered to have the same immunoglobulin fold.

It is important to realize that, as with sequence comparisons, structural similarities form a continuum. Cut-off points for acceptable levels of structural similarity for related folds were derived by empirical trials¹⁰ on proteins having distinct or common folds. Two structures were deemed to adopt the same topology if their SSAP score was more than 70. To ensure global similarity, at least 70% of residues in the larger protein should be matched to residues in the smaller.

Fold classification

On the basis of sequence alignment, followed by clustering together of structures with more than 25% sequence identity, we find that the 2,511 polypeptide chains, determined by X-ray crystallography to <3.0 Å, cluster into 212 sequence families. Subsequent structural comparisons between representatives from each of these sequence families allowed further clustering on the basis of significant SSAP scores. This gave 80 single-

domain fold families. Therefore a low sequence similarity ($<25\%$) need not imply that the protein will have a novel fold. Given a completely novel sequence ($<25\%$ identity to any other sequence) there is at present only a 30% probability that the protein will possess a novel fold (Fig. 1). As remote homologues are difficult to identify from sequence alone, this figure represents an accurate view of the current data and the probability of finding a novel fold if only sequence data are available.

Extension of classification

The 25% sequence identity cutoff does not eliminate all homologues (for example, some globins have $<15\%$ sequence identity). More sophisticated template-based methods would detect some, but not all, of these remote relationships. To remove such homologues, we used structural comparisons giving SSAP scores >70 , combined with observation of a common function, which generally indicates divergence from a common ancestor. Two proteins are deemed to have a common function on the basis of rather broad categories that are reflected in their structural similarity. For example, the up-down β -barrels, which all bind hydrophobic ligands at one end of the barrel, are classed as one functional family, but the cytokine interleukin-1 β , soybean trypsin inhibitor and ricin are proteins with different functions. We call these enlarged families hyperfamilies to distinguish them from the superfamilies, reducing the number of sequence families from 212 superfamilies to only 131 hyperfamilies. Thus, if sequences are grouped into hyperfamilies, the probability that a non-homologous sequence ($<25\%$ sequence identity and no functional similarity) will possess a novel fold increases to 53%.

Statistical evaluation of fold numbers

Given that structures do recur, it is reasonable to ask how many fold types exist in nature. Owing to different stereochemical constraints (the packing together of helices and strands), the number of ways that a sequence can fold may be limited^{11,12}, and has been estimated at $<1,000$ folds¹³. We used a statistical sampling approach based on our structural comparison data to determine the number of protein folds.

If we assume that (1) there are N fold structures, which are equally probable; (2) proteins have been selected for structural determination at random from the pool of all proteins; and (3) from this group of proteins we only compare the structures of those with no statistically significant sequence similarity ($<25\%$ sequence identity); then for any one comparison the probability of two proteins having the same fold is $1/N$, where N is the total number of folds. Making structural comparisons over the complete databank of sequentially unrelated structures, the number of matches is then evaluated as the number of comparisons $\times (1/N)$. We have excluded multidomain proteins from this analysis as it is difficult to estimate the number of structural matches between these proteins without knowing the domain boundaries. From our classification of structures, we have 212 proteins with unrelated sequences ($<25\%$) giving $(212 \times 211)/2$

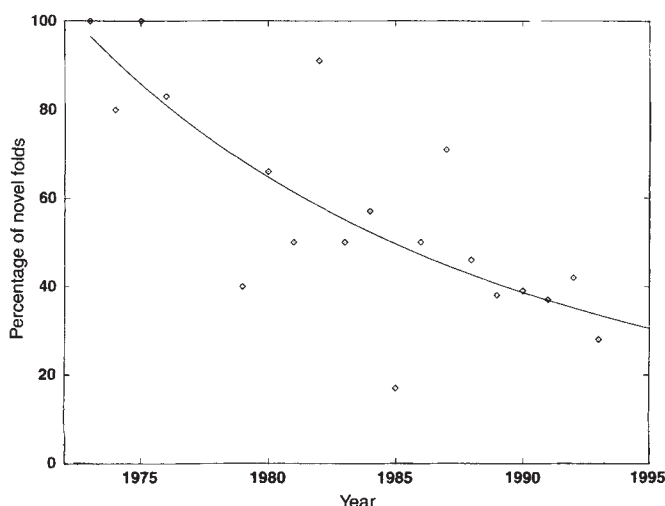


FIG. 1 Annual decrease in the percentage of newly-determined protein structures which have novel folds, as found by X-ray crystallography and NMR techniques. This is expressed as a percentage of the number of non-homologous structures (<25% sequence identity) for a given year.

comparisons. We observed 583 matches (counting each match once and ignoring all self against self-matches). Using these values, N is predicted to be 38 folds. As we already know of 80 single-domain folds, some of the assumptions above must be incorrect. If we use our improved classification of 131 non-homologous folds, we observe 191 matches, which still predicts only 45 folds.

This suggests that our assumption that all N folds are equally populated is not valid. The distribution of structures between the different fold families is illustrated in Fig. 2. It is far from what might be expected to have arisen by chance. In summary, we observe nine fold families, for which there are between 3–11 examples of unrelated sequences with unrelated functions, and 71 folds for which there is only one example (singlets). If this were a random sampling of a set of folds, each of which were equally probable, in this number of observations we would expect to see mainly singlets, a few doublets and then decreasing numbers of multiplets. Clearly, the distribution is very skewed from this all-equal hypothesis (Fig. 2). It is dominated by the

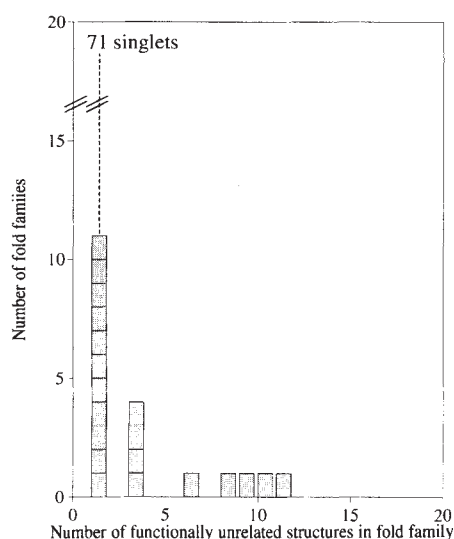


FIG. 2 Distribution of current non-homologous structures (<25% sequence identity and <80 SSAP and no functional similarity) among the different folds. The nine superfolds contain between 3 and 11 representatives, whereas all the remaining folds are singlets.

nine superfolds (a superfold being defined as one for which we have already observed three or more non-homologous structures). In our database with 131 hyperfamilies, if we assume all folds are equally populated and there are about 1,000 folds, the expected number of triplets (three non-homologous domains with the same fold) is 0.32, four matches is 0.012 and ten matches (as found for the TIM-barrel motifs) is 4.1×10^{-13} . In addition, for most of these superfolds, there are multiple homologous structures that have the same function but for which the sequences show no similarity.

The observation that all fold types are not equally probable is not new, but now with the benefit of many novel structures, it is apparent that the observed distribution is very skewed. Thus, although many proteins (32% of all database, and 46% of non-homologous proteins) are found to adopt one of the nine superfolds, there are also many proteins with fold types seen only once. For example, the folds of lysozyme and ribonuclease, despite being the first enzyme structures to be solved, have not been observed elsewhere in non-homologous proteins (that is, proteins with the same fold, but no sequence or functional similarity).

The existence of the superfolds (such as TIM barrels or Greek-key proteins; Table 1 and Figs 2, 3) therefore distorts the match rate in our statistical analysis, making it much higher than expected for an 'equally populated fold model'. By considering only matches other than within the superfolds, the method could still be applied, but we have not yet observed any such matches, suggesting that the minimum number of fold types is greater than 1,000. If the number were substantially less, then we would expect to have observed a few doublet matches other than between the superfolds.

TABLE 1 Superfolds

Fold	Structures (PDB code)
α/β doubly wound	11 Ras p21 (5p21), flavodoxin (4fxn), carboxypeptidase (5cpa), elongation factor-Tu (1etu), subtilisin carlsberg (1cseE), phosphoglycerate mutase (3pgm), thioredoxin (2trxA), chey (3chy), glycinamide ribonucleotide transformylase (1grcA), adenylate kinase (3adk), dihydrofolate reductase (4dfrA)
TIM barrel	10 triosephosphate isomerase (5timA), mandelate racemase (1mnr), aldolase (1ald), anthranilate isomerase (1pii), tryptophan synthase (1wsyA), Rubisco (5rubA), D-xylose isomerase (1ximA), taka-amylase A (2taaA), enolase (4eni), glycolate oxidase (1gox)
Split $\alpha\beta$ sandwich	9 ferredoxin (1fxd), crambin (1crn), L7/L12 ribosomal protein (1ctf), acylphosphatase (1aps), histidine-containing phosphocarrier (2hpr), procarboxypeptidase (1pba), DNA-binding protein (1bop), N-terminal fragment of nuclear ribonucleoprotein (1nrcA), ribulose (S chain) (3rubS)
Greek key-immunoglobulin	8 Bence-Jones protein (2rhe), macromycin (2mcm), CD4 (2cd4), telokin (1tik), prealbumin (2pabA), superoxide dismutase (2sodB), tenascin (1ten), human class I histocompatibility antigen (1hsaB)
α -up-down	6 hemerythrin (2hmqA), cytochrome B562 (256bA), apolipoprotein E3 (1lpe), tobacco mosaic virus (2tmvP), granulocyte-macrophage colony-stimulating factor (1gmfA), ligand-binding domain (1lig)
Globin	3 erythrocyruorin (1eca), colicin A (1colA), phycocyanin (1cpcA)
Jelly roll	3 tobacco necrosis virus (2stv), tumour necrosis factor (1tnfA), pea lectin (2ltnA)
Trefoil	3 interleukin-1 β (1i1b), ricin (1aaiB), erythrina trypsin inhibitor (1tie)
UB $\alpha\beta$ roll	3 ubiquitin (1ubq), ferredoxin I (1fxiA), protein G (1pgx)

The nine fold families in the current dataset that are highly populated with functionally unrelated non-homologous proteins. For each family the numbers of functionally unrelated representatives are given (middle column), together with their titles and Brookhaven PDB codes in parentheses.

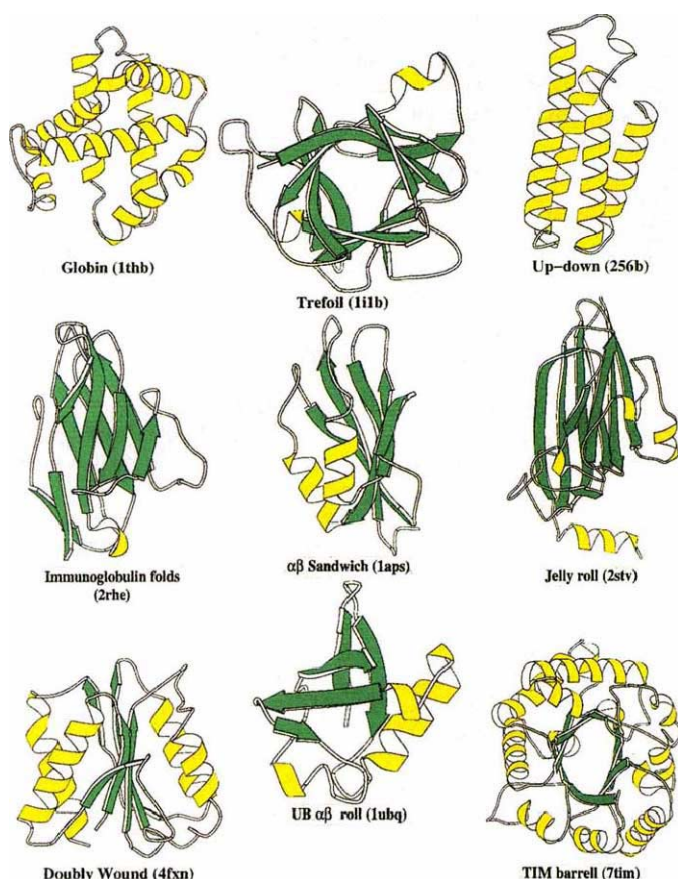


FIG. 3 MOLSCRIPT²⁷ representations of the nine protein domain superfolds.

The number of superfamilies

There is, of course, an upper limit to the number of unique protein fold structures, as there can be no more protein folds than protein sequences, and given that homologous sequences are known to have identical folds, there can be no more folds than protein families. Given the difficulties in identifying remote homologies, it is hard to produce an accurate count of the number of known protein families. Dayhoff and colleagues¹⁴ divided sequences into families and superfamilies: families of sequences sharing sequence identities >50% and superfamilies sharing sequence identities >30–40%. Superfamilies can be grouped together by exploring sequence similarities below 30%: for example myoglobin and haemoglobin sequences form separate superfamilies, but are clearly related below the 30% level. However, clustering sequences at low levels of sequence similarity is prone to error owing to poor statistical significance. In the face of these problems, we opted to analyse the sequence databank at the superfamily level, rather than use more sensitive profile methods to identify more distant relationships.

To determine the present number of known superfamilies, we extracted sequences from the SWISS-PROT database, release 27, that were between 30 and 1,500 residues long (about 28,000 sequences in total). Using the FASTA program¹⁵, all pairwise comparisons were made to identify any pairs of sequences that could have sequence similarity when aligned using a more rigorous method. All transmembrane sequences were removed. To identify every possible match, a very low cutoff of 100 was used on the FASTA score for this process. Every sequence pair giving a greater FASTA score was then subjected to a full global alignment¹⁶ to determine an accurate level of sequence identity. Finally, sequence pairs with more than 30% sequence identity were clustered together into superfamilies (30SEQ families) using an efficient single linkage clustering algorithm. This pro-

cess revealed a total of about 7,700 superfamilies. Figure 4 shows that this number is still increasing rapidly, although the ratio of the superfamilies/sequences is approximately constant, and if anything may be decreasing. Our result represents an upper limit to the number of naturally occurring fold types in the present sequence databank.

Number of folds re-estimated

With this estimation of the number of superfamilies, it is now possible to recalculate the total number of fold types, using the approach of Chothia¹³, but incorporating these estimates derived from our more detailed analyses of the sequence and structural data. In the SWISS-PROT database, our analysis shows that there are currently 28,000 non-transmembrane sequences, with examples from all phyla, which reduce to 7,700 superfamilies at the 30SEQ level of comparison. As roughly one-third of all new sequences are found to be homologous to old sequences at this 30SEQ level, it has been suggested that we already have representatives for one-third of all naturally occurring superfamilies¹³. This implies that there is a total of 23,100 protein superfamilies.

In the structural database we have 234 30SEQ single-domain superfamilies, reducing to 131 hyperfamilies and then to 80 single-domain fold structures. There are also 65 multidomain structures which we have not considered here. Thus in the structural database at the moment we only have representatives for 234 of the currently known 7,700 30SEQ superfamilies (roughly 3%). This figure is much smaller than the estimated 25% of sequences¹⁷ in the SWISS-PROT database for which the structure of a homologue is known. This reflects the bias in the current sequence databases towards some families (for example, the immunoglobulins) and emphasizes the importance of considering sequence families rather than individual sequences.

Thus 3% of the current sequence databases (in terms of superfamilies) has yielded 80 domain folds. If the sequences represent only one-third of all superfamilies, the total number of domain folds in all phyla can be estimated as $80 \times 33 \times 3 = 7,920$. This figure is much larger than the 1,000 folds estimated¹³, and results from the consideration of sequence superfamilies rather than just sequences. However, this figure is a considerable overestimate for the following reasons, which must be considered when trying to estimate the number of folds. Many of the sequences represent large multidomain proteins and some are polypeptides. From the different domain folds, many different multidomain proteins can be constructed by combining different domains, as recently exemplified by the β -galactosidase structure¹⁸, which has five domains, including three superfolds (one TIM barrel; two immunoglobulin-like folds and a jelly-roll). Thus, we expect that many of the larger proteins in SWISS-PROT will consist of combinations of the basic domain folds. The other important

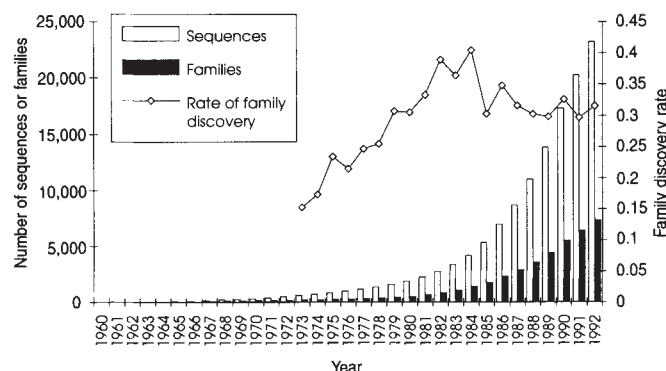


FIG. 4 Cumulative increases in the number of sequences (white bars) and sequence families (black bars) against year. The line shows the rate of family discovery, given by dividing the number of new sequence families by the number of new sequences in each year.

consideration is the assumption that the current structure database is a random selection of all natural protein sequences (so that we can apply the factors derived from the structural database to the sequence data), which is debatable. These considerations, combined with the problems of the structural continuum, render such calculations of the total number of fold types very imprecise. But it is clear that currently we only know the structure for about 1% of all naturally occurring 30SEQ superfamilies (3% of known superfamilies), although this percentage will be different for the hyperfamilies.

Discussion

This analysis has highlighted the unequal population of naturally occurring protein folds. The distribution we observe may simply represent a bias in the sampling of proteins for structural determination, but the odds against observing such high multiplets by chance are overwhelming, thus almost certainly, the fold families are not equally populated. Such a possibility has been suggested by other groups^{7,19,20} and consideration of some of the classic architectures long ago, showed a striking preference for some topologies rather than others. For example, with 4- α bundles the up-down-up-down packing dominates the observed structures, whereas the up-up-down-down fold is only found in a single functional family (the growth hormones). With the $\alpha + \beta$ sandwiches, only 6 of the 22 possible topologies were observed and two of these accounted for 50% of the non-homologous representatives²¹.

The existence of superfold structures has several implications. Given that no structural matches are observed outside the superfolds, it is currently not possible to estimate reliably the total number of folds using a statistical approach, as we had originally envisaged. For the recognition of fold types from sequence, using threading or profile methods^{22,23}, the target folds are not all equally likely to occur. A new sequence (<25% sequence identity) may adopt one of the superfolds, or if the functions are similar, it may be related to a singlet protein of known structure. Thus fold prediction by recognition should concentrate on the superfolds or structures with related functions. But there are also some examples where sequentially homologous proteins have remarkably different functions (for example, enzymes used as structural proteins in the eye lens²⁴, or α -lactalbumin and lysozyme, which have >30% sequence identity). Thus one protein can be utilized for a completely different function, even when it is closely related from an evolutionary and sequence viewpoint. These observations raise several important questions. (1) What distinguishes a superfold from a singlet structure and why are there superfolds? (2) Do the superfolds represent convergent or divergent evolution? (3) How long will it be before we have observed all naturally occurring folds? (4) To what extent has nature exploited all possible folds?

These superfolds may represent extra stable folds that have diverged from a common ancestor and despite extensive changes in sequence, the topology remains the same. Any residual sequence patterns are usually completely undetectable, and the original function may also have changed. Alternatively, these superfolds may reflect the accidental convergence of disparate polypeptide chains with diverse functions towards the same topology. Whatever the origin of the superfolds, the observations based on sequences and structures suggest that a superfold is compatible with a much larger set of sequences, compared with one of the singlet folds (see also ref. 25).

It is striking that many of the superfolds have very simple topologies, with a high percentage of sequential secondary structures, such as TIM barrels, which lie adjacent in the tertiary fold. It is tempting to suggest that one of the contributory factors determining the high frequency of these folds is that, for them, kinetic folding is straightforward compared with some of the more complex folds. In an all-sequential structure, it is apparent that, in principle, folding could begin anywhere and proceed in any direction; there are no obligate pathways, but many routes

to the same folded structure. If this hypothesis is correct, we would expect that the all-sequential up-down β -barrels and the β -propeller structures (for example, neuraminidase) should also be superfolds, which looks to be the case for the β -propeller structures. Similarly, in the $\alpha + \beta$ class, the OB²⁶ folds and the meander α - β sandwiches²¹ also have a simple all-sequential topology, and may be superfolds. Many of the singlet structures are relatively small and held together by disulphides or metals. These provide additional stabilizing crosslinks (most disulphide-bonded proteins denature if the disulphides are broken). This adds sensitive hot-spots where mutations might be lethal. Without these additional constraints, which are highly conserved, these folds might not be sufficiently stable to withstand random mutation.

The diverse functions of the superfold structures suggests that they may have arisen independently and not by divergent evolution. This is especially true for the small regular domains (such as the up-down-up-down 4 α -bundles and the $\alpha + \beta$ sandwiches). The argument is harder for the TIM barrels because they are mainly found in enzymes with their active sites located at the C-terminal ends of the strands in the β -barrel. Similarly, the immunoglobulin fold is employed in many different guises in the immune system. Perhaps these proteins were simply present in the extracellular medium and were co-opted to serve many functions. However, the immunoglobulin-like enzymes and copper-binding proteins (for example, superoxide dismutase), which have a related fold, may have arisen independently.

Given the distribution of folds, it is clear that although many proteins adopt a superfold, a large fraction are singlets, having their own unique structure. These singlet structures must arise only very rarely by chance and therefore, until we have determined the structure of all the protein families, we may not have seen all the naturally occurring folds.

It is unlikely that nature has exploited all possible fold structures, which offers wide scope for the designing proteins with new topologies. However, the fold distribution also suggests that it should be less difficult to construct a completely novel sequence for a superfold than a singlet structure. Designing a stable fold that has not yet been found may be much more challenging. □

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An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita

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X-linked adrenal hypoplasia congenita is a developmental disorder of the human adrenal gland that results in profound hormonal deficiencies and is lethal if untreated. We have isolated the gene responsible for the disease, *DAX-1*, which is deleted or mutated in X-linked adrenal hypoplasia patients. *DAX-1* encodes a new member of the nuclear hormone receptor superfamily displaying a novel DNA-binding domain. The *DAX-1* product acts as a dominant negative regulator of transcription mediated by the retinoic acid receptor.

ADRENAL hypoplasia congenita is an inherited disorder of adrenal gland development. Two distinct forms, the autosomal recessive miniature and the X-linked cytomegalic form, have been described¹. X-linked, adrenal hypoplasia congenita (AHC; MIM 300200)² is characterized by the absence of the permanent zone of the adrenal cortex and by a structural disorganization of the glands. The disorder, which is lethal if untreated, results in adrenal insufficiency early in infancy, with low serum concentration of glucocorticoids, mineralocorticoids and androgens, and failure to respond to adrenocorticotrophic hormone (ACTH) stimulation. Hypogonadotropic hypogonadism (HHG) is commonly associated with the X-linked form of the disease and is generally noted at the expected time of pubertal maturation. It is not clear if this form of HHG is of pituitary or hypothalamic origin (reviewed in ref. 3).

AHC has been mapped to chromosome Xp21 on the basis of the identification of several patients with deletions in this region and clinical signs of a contiguous gene syndrome that may include AHC, HHG, mental retardation, glycerol kinase deficiency (GKD) and Duchenne muscular dystrophy (DMD)⁴⁻⁶. The region encompassing the DMD, GKD and AHC loci has been cloned in yeast artificial chromosomes (YAC) and the AHC locus mapped to a 250–500 kilobase (kb) region, telomeric to the GKD locus⁴⁻⁶.

We have recently mapped an X-linked locus involved in sex determination, DSS (for dosage-sensitive sex reversal) to Xp21. Patients with an intact *SRY* gene and duplications of this chromosomal region develop as phenotypic females. The characterization of patients with microscopic duplications of Xp21 and the identification of a submicroscopic duplication in one 46,XY sex-reversed female allowed mapping of the DSS locus to a 160 kb region, adjacent to the AHC locus⁷.

To clone the gene(s) underlying DSS and AHC we isolated expressed sequences from a phage contig spanning the region.

The gene we describe here, *DAX-1* (for DSS-AHC critical region on the X chromosome, gene 1) is deleted in AHC deletion patients and mutated in AHC non-deletion patients, indicating that functional deficiency for the gene is indeed responsible for the disease. The C-terminal 250 amino acids of the protein encoded by *DAX-1* (*DAX-1*) show roughly 50% continuous similarity to the ligand-binding domain of members of the nuclear hormone receptor superfamily. Strikingly, the amino-terminal portion contains four incomplete repeats of a new structural motif that bears DNA-binding function. We show that *DAX-1* binds to retinoic acid (RA) responsive elements and downregulates RA receptor-mediated transcriptional activation.

Isolation of the *DAX-1* gene

The DSS, AHC, GKD and DMD loci were previously shown to be closely linked in Xp21⁴⁻⁶. To identify expressed sequences from the DSS-AHC region, we analysed, by pulse-field gel electrophoresis (PFGE), previously identified YAC clones⁶. A single CpG-rich region was identified within the portion of the DSS-AHC critical interval covered by these YACs (not shown). The overlapping YACs A107E5 and 332F10⁶ were subcloned in λ FIXII vector and a 230 kb phage contig was constructed. The CpG-rich region was localized on the phage contig by *Eag*I, *Bss*II and *Sac*II digestion (Fig. 1a). A 1.6 kb *Sac*I fragment spanning the CpG-rich region was subcloned in the plasmid vector and shown to be conserved during evolution by hybridization to a 'Zoo blot' (not shown) and to include portions of expressed sequences by northern blot analysis (not shown). The 1.6 kb *Sac*I fragment was used for the screening of complementary DNA libraries obtained from human adult testis and human fetal adrenal RNA. Several overlapping cDNA clones, corresponding to a single gene, *DAX-1*, were isolated.

Structure of the *DAX-1* gene

Figure 1b illustrates the overlap of the *DAX-1* cDNA clones, as determined by restriction mapping, polymerase chain reaction (PCR) and sequence analysis. The positions of *DAX-1* exons were identified in the genomic DNA by Southern blot hybridiza-

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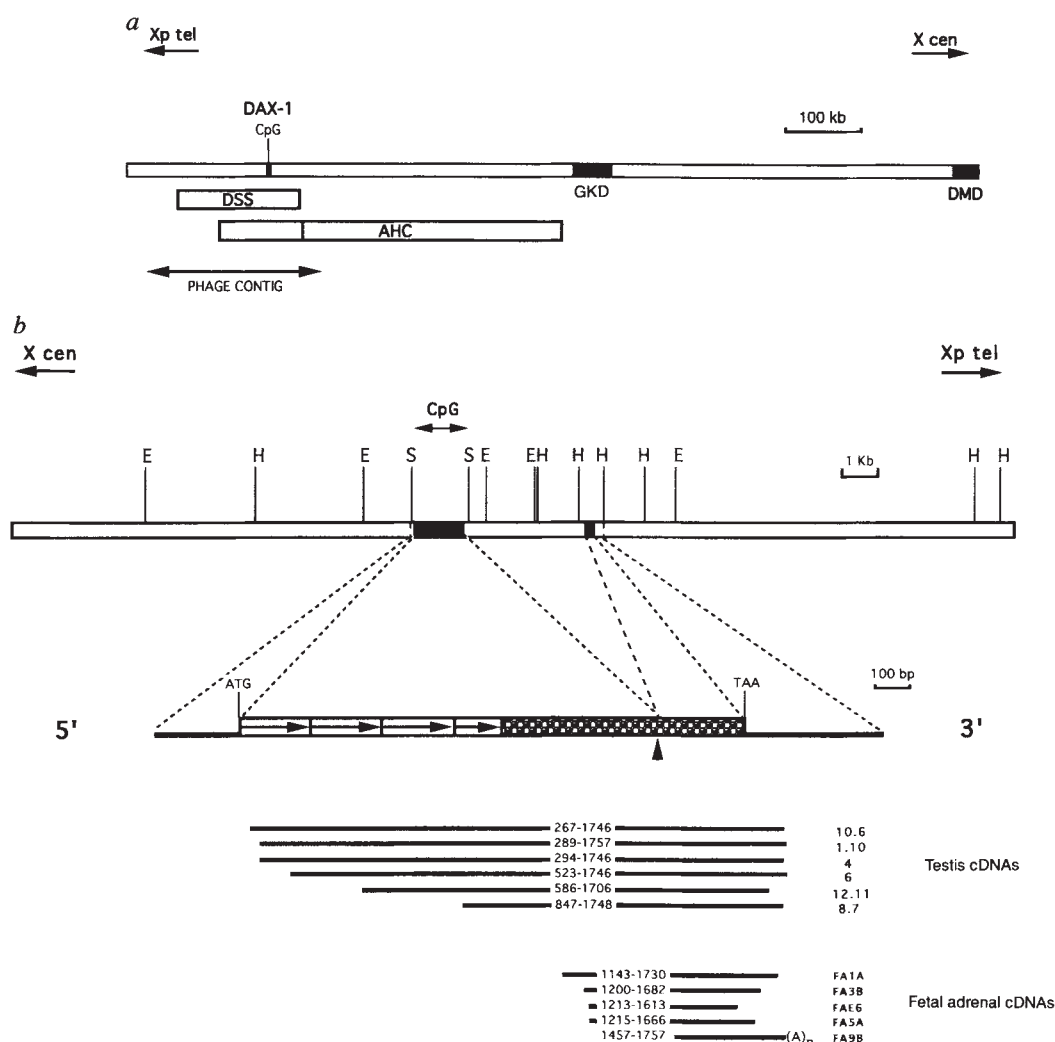


FIG. 1 Isolation of the *DAX-1* gene. **a**, Map of the DSS and AHC critical regions in Xp21. The localization of the GKD locus, of part of the DMD locus and of the CpG island described in the text are shown. The DSS and AHC critical regions are represented by open boxes. The bar within the AHC critical interval represents the distal breakpoint of patient T.M.⁷. **b**, Top, genomic organization of *DAX-1*. Black bars, coding portions of *DAX-1*. E, *EcoRI*; H, *HindIII*; S, *SacI*. The orientation with respect to the Xp telomere is opposite to the one in **a**. Centre, organization of the *DAX-1* mRNA as deduced from sequence analysis. The arrow shows the position of the single intron. Bottom, schematic representation of the *DAX-1* cDNAs isolated from human adult testis and human fetal adrenal cDNA libraries. The numbers on the cDNAs corresponds to the nucleotide numbers in Fig. 2a. The 5' longest cDNA we isolated (cDNA 10.6, **c**) starts at position 267 and does not contain the presumptive initiation codon. Three lines of evidence suggest, however, that the ATG in position 235 is the initiation codon. (1) The length of the mRNA, as deduced from northern blot hybridization experiments (Fig. 4a), does

not exceed 1.9 kb. This indicates that cDNA 10.6 is nearly full length. (2) RT-PCR analysis demonstrated that the ATG in position 235 is transcribed in both adult testis and adrenals (not shown). (3) Ribosomes preferentially initiate translation at the first ATG codon in a good context³⁷. Both the nucleotide sequences flanking the ATG codon in position 235 and those flanking the second ATG codon in position 280 fulfil Kozak's criteria³⁸ for an authentic initiation codon. **METHODS.** Digestion of DNA with restriction enzymes and Southern blot analysis were all according to standard procedures³⁹. Total DNA was isolated from the YAC clones according to ref. 40 and PFGE was as described⁴¹. Construction of the λ phage libraries from the YACs and contig construction were as described⁴². Screening of the cDNA libraries was according to standard procedures³⁹. Plaque-forming units (10^6 PFU), of the human adult testis (Clontech) library were screened with the 1.6 *SacI* fragment and rescreened with cDNA 10.6. cDNA 10.6 was used to screen 10^6 PFU of the fetal adrenal gland library (Clontech).

tion and restriction enzyme analysis. The cDNAs and the corresponding genomic DNA fragments (1.6 kb *SacI* and 0.7 kb *HindIII* fragments, Fig. 1b) were sequenced.

The sequence of 1,757 base pairs (bp) of the cDNAs and 5' and 3' genomic flanking sequences is shown in Fig. 2a. The sequence contains a single long open reading frame that starts with an ATG at nucleotide 235 and ends with a TAA stop codon at nucleotide 1,645, giving a predicted protein product of 470 amino acids. The coding sequence is split by a single intron of about 3 kb, located between nucleotides 1,402–1,403 (Figs 1b and 2a).

The predicted amino-acid sequence for *DAX-1* was used as a query to search the non-redundant protein sequence database

of the National Center for Biotechnology Information using the BLAST network service⁸. The analysis revealed a highly significant similarity between the C-terminal half of *DAX-1* and the ligand-binding domain (domain E) of the nuclear hormone receptor gene superfamily. The best homology scores were observed between the *DAX-1* C-terminal domain and the E domain of the retinoid X receptor (RXR) subfamily^{9,12} and of the orphan receptors (EAR-2¹³, seven-up¹⁴, COUP^{15,16}, ARP-1¹⁷) subfamily (Fig. 2b). Lower but significant similarity scores were observed with the other members of the nuclear receptor gene superfamily.

No sequence with significant homology was found for the first 253, N-terminal amino acids of the predicted protein, even when

used alone (without the C-terminal part) as a query to search in the database. This N-terminal domain, which is very rich in small amino acids (glycine and alanine), can be organized in three and a half repeats of a 65–67 amino acid motif with cysteines in conserved positions (Fig. 2c). The structures of the *DAX-1* putative protein product and the mouse RXR γ are compared in Fig. 2d.

The *DAX-1* gene was oriented with respect to the X short arm, with the 3' end located toward the telomere and the 5' end toward the centromere (Fig. 1b). Low stringency Southern blot analysis failed to reveal sequences closely related to *DAX-1* (see lanes 1, 2 and 14, 15 in Fig. 4c).

Analysis of patients

The study of patients with deletions or duplications in Xp21 demonstrated that the AHC and DSS critical intervals are closely linked^{4–7}. To define the possible role of the *DAX-1* gene in gonads and/or adrenal gland development, we analysed both sex-reversed 46,XY female patients and AHC male patients with deletions in Xp21.

The DNA of thirty 46, XY female patients, previously shown not to be duplicated for this chromosomal region⁷, was digested with *Eco*RI, *Pst*I or *Taq*I, and hybridized with the cDNA 10.6. In all cases we observed a normal hybridization pattern (not shown).

Southern blot analysis of AHC deletion patients provided, however, a strong indication for the involvement of *DAX-1* in X-linked adrenal hypoplasia congenita. The cDNA 10.6 was hybridized to all key patients used, by two different groups^{4,6}, to define the AHC critical interval. *DAX-1* was found to be deleted in all these patients, with the exception of one (patient T.M.^{6,7}; Fig. 3a and results not shown).

To prove that *DAX-1* is responsible for AHC, patients with no detectable deletions or rearrangements in this region were analysed for the presence of mutations in the coding portion of the *DAX-1* gene. Patients' DNAs were amplified using primers specific for *DAX-1* and the PCR products were directly sequenced. A deletion of 11 base pairs (including nucleotides 983–993 in Fig. 2a), resulting in a frame shift and premature termination, was identified in two brothers, M.I.N. and M.E.N.¹⁸, affected by AHC and HHG (Fig. 3b). A substitution of G to A at nucleotide 938, creating a nonsense mutation at codon 235 (TGG-TGA) and a new *Mae*I site, was identified in patient B.F., who is also affected by AHC and HHG (Fig. 3c). Finally, a 4 bp insertion (ACCC) at nucleotide 779, resulting in a frame shift and premature termination and in the creation of a new *Sma*I site, was identified in patient 2957 and in his carrier mother (Fig. 3d). These data establish that the absence of an intact *DAX-1* gene product is responsible for both AHC and HHG. It is thus likely that the deletion in patient T.M., involving a region 5' to the *DAX-1* gene (Figs 1a and 3a), inhibits the correct expression of the *DAX-1* gene.

Pattern of expression and conservation

To determine the tissue distribution of *DAX-1* gene expression, total RNA from human adult and fetal tissues and from cultured cell lines was transferred to a nylon filter and hybridized with cDNA 10.6 (Fig. 4a, b). The distribution of *DAX-1* messenger RNA reveals a very specific pattern, with detectable expression in adult testis, adult adrenals and in the teratocarcinoma cell line. No expression was detected in the remaining tissues and cell lines, even after prolonged exposure of the blot. Reverse transcription-PCR revealed low levels of *DAX-1* expression in adult ovary and liver (not shown). The human *DAX-1* mRNA appears as a single 1.9 kb RNA species.

We studied the conservation of *DAX-1* during evolution by Southern blot hybridization of cDNA 10.6 to a 'Zoo blot' containing DNAs from male and female individuals belonging to different species (Fig. 4c). The probe detects X-linked fragments in human DNA digested with *Hind*III or *Eco*RI, and homolo-

a	GAGCTCCACGCTGCTGTTCTTCCATTTCACGCTTTTAAAGAGCACCCGCCCT	54
	TCGAACACACGAGGTCATGGGCGAACACACCGAGCGCAGACCGCCGCCCCCGCACACA	114
	CCGCCCGCCTCCGCGCCCTTGCCAGACCGAGGCGCGCAGCGCCTGCGTGCCTAG	174
	GTATAAATAGTCCAGGAGGCGAGCTGGGCGAAGCTGGGCTACGGCGCGCCGGGGC	234
	ATGGCGGGCAGAGAACCCAGTGGCAGGCGAGCATCTCTACAACATGCTTATGAGCGCG	294
	M A G E N H Q W Q G S I L Y N M L M S A	20
	AAGCAACGCGCGCGCTCTGAGGCTCCAGAGACGCGGCTGGTGGTACAGTGTGGGGC	354
	K Q T R A A P E A P E T R L V D Q C W G	40
	TGTTCTGTCGGCGATGAGCCCGGGTGGGCGAGAGGGGCTGCTGGGCGGGCGAACGTG	414
	C S C G D E P G V G R E G L L G G R N V	60
	GCGCTCTGTACCGCTGCTGCTTTTGGGTAAAGACACCCAGGCGAGGCGAGCATCTCTC	474
	A L L Y R C C F C G K D H P R Q G S I L	80
	TACAGCATGCTGACGAGCGCAAGCAACGTCAGCGGCACGAAGGCGCCGAGGCGACG	534
	Y S M L T S A K Q T Y A A P K A P E A T	100
	CTGGGTCGCTGCTGGGCTGTTCTGCGGCTCTGATCCCGGGTGGGCGAGCGGGGCTT	594
	L G P C W G C S C G S D P G V G R A G L	120
	CCGGTGGGCGGCGCTGGCACTCTGTACCGCTGCTGCTTTTGGTGAAGACACCCG	654
	P G G R P V A L L Y R C C F C G E D H P	140
	CGGCGGGCAGCATCTCTACAGCTTGCTCACTAGCTCAAGCAACGACGCTGGCTCCG	714
	R Q G S I L Y S L L T S S K Q T H V A P	160
	GCAGCGCCGAGGCGCGGCGAGGGCGCGTGGTGGGACCGCTCTACTTCGCGCAGAGG	774
	A A P E A R P G G A W D R S Y F A Q R	180
	CCAGGGGTAAAGAGGCGCTACAGGCGGGCGGCCAGGCGCTCTGTACCGCTGCTGC	834
	P G G K E A L P G G R A T A L L Y R C C	200
	TTTTCGGTGAAGACACCCGAGCAGGCGCAGCCCTCTACTGCTGCCACGAGCACA	894
	C G E D H P Q Q G S T L Y C V P T S T	220
	AATCAAGCGCAGCGGCTCCGAGGAGCGCGCCGAGGCGCCCTGGTGGGACACCTCTCT	954
	N Q A Q A A P E E R P R A P W W D T S S	240
	GGTGCCTGCGGCGGCTGCGCTCAAGAGTCCAGAGTGGTCTGCGAGGCGCCTCAGCG	1014
	G A L R P V A L K S P Q V V C E A A S A	260
	GGCTGTTGAAGACGCTGCGCTTCGTCAGTACTTGCCTGCTTCCAGGCTGCTGCCCTG	1074
	G L L K T L R F V K Y L P L C Q V L P L	280
	GACCAGCAGCTGGTGTGGTGCACACTGCTGGGCGTCCCTGCTCATGCTGAGCTGGCC	1134
	D Q Q L V L V R N C W A S L L M L E L A	300
	CAGGACCGCTTGCACTTCGAGACTGTGAAGTCTCGAGGCCAGCATGCTCGAGAAGATC	1194
	Q D R L Q F E T V E V S E P S M L Q K I	320
	CTCACCACCGGCGGGGAGACCGGGGCAACGAGCCAGTGGCCGTGCCACGCTGCAG	1254
	L T T R R E T G G N E A P L P C F V L T Q	340
	CACCATTTGGCACCAGCGGCGAGGCGAGGAGTGGCTCCGCTCCAGGTTCAAGCC	1314
	H H L A P P A E A R K V P S A S Q V Q A	360
	ATCAAGTGTCTTCTTCCAAATGCTGGAGTCTGAACATCAGTACCAAGGAGTACGCTAC	1374
	I K C F L S K C W S L N I S T K E Y A Y	380
	CTCAAGGGACCGTCTCTTTAACCCGAGCTGCGGCGCTGCGTGAAGTACATT	1434
	L K G T V L F N P D V P L G C Q V K Y I	400
	CAGGACTCCAGTGGGAACTCAGCAATACTAGTGAACACACAGGATGACGCCACAA	1494
	Q G L Q W G T Q Q I L S E H T R M T H Q	420
	GGGCCCCATGACAGATTCATGAACCTTAATAGTACCTTTTCTGCTGAGATTCATCAAT	1554
	G P H D R F I E L N S T L F L R F I N	440
	GCCAATGTCATTGCTGAACCTTCTTCAGGCCCATCATCGCACAGTACGATGATGAT	1614
	A N V I A E L F R P I I G T V S M D D	460
	ATGATGCTGGAAATGCTCTGTACAAAGATATAAAGTCATGTGGGCCACACAAGTGCAGTA	1674
	M M L E M L C T K I *	470
	GTGCAGTTCCACATGAGGGAAGATAAGAGCTGTGGGCAAGAGTGTAATAATTTT	1734
	AAATAAACTTTCTTAATATTTTACATGACAGATATTTTGATCTTCAATTAAAGAAATAA	1794
	TTTATTCCCGACGACGATCAAAATTTCTGTTCATAGTTAAAGAGACATTTGCCAA	1854
	CAGGTAGCATAGCTCTGTACATCTTTTAAAAAATTCGAGGCTACTAGTATAATAAG	1914
	CTATTTCACAAGCGCAGCAATTTTCATGGAACCTGCTCAATCAATTTGTACATATTGT	1974
	TATAATAAAATTTAAGGCTCTTAACATTAACTTGATTGAAAAAGCTT	2022

FIG.2a See next page.

gous fragments in all the species tested, including chicken DNA, but not in *Drosophila melanogaster*. Dosage analysis suggests that the crosshybridizing fragments in mammals are X-linked.

The *DAX-1* protein is nuclear

In order to study the function of the AHC gene product, we inserted the full-length cDNA into the bacterial expression

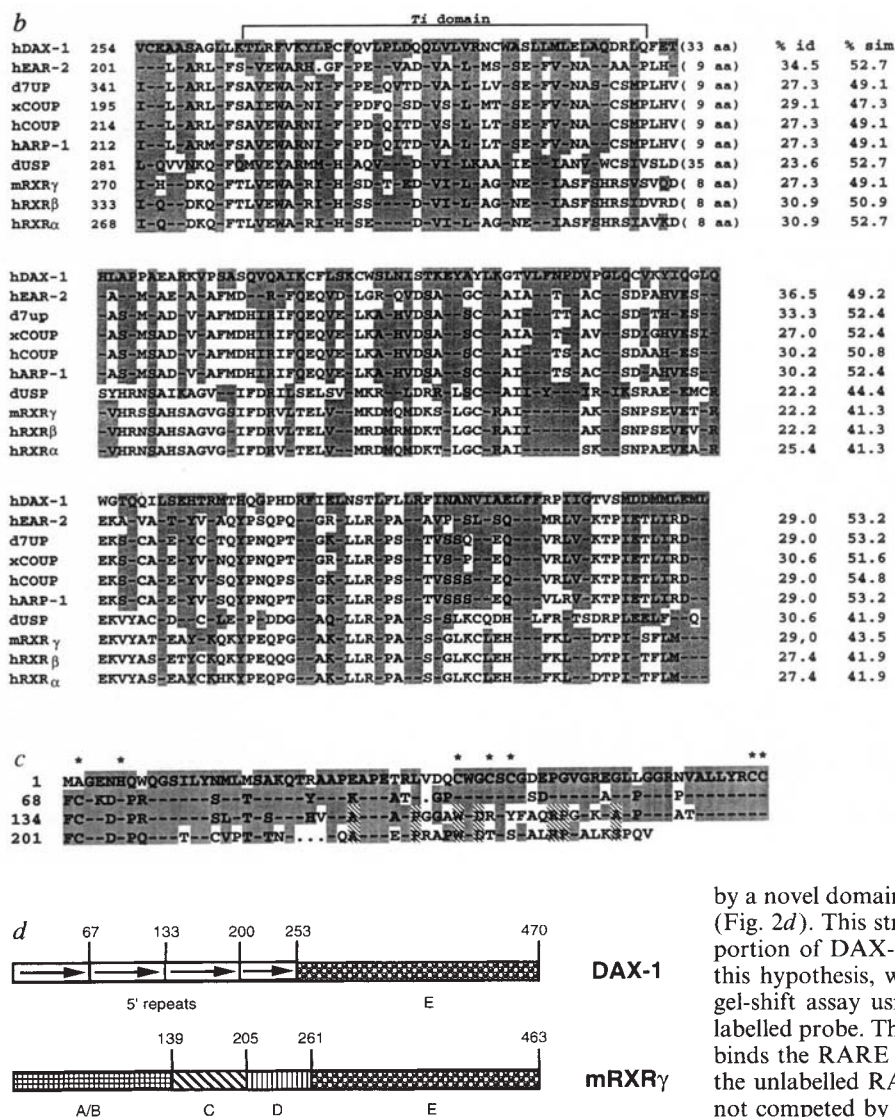


FIG. 2 Characterization of the *DAX-1* sequence. **a**, Nucleotide and predicted amino-acid sequence of *DAX-1*. The position of the intron and the sequence of the splice sites is shown above the sequence. Three potential polyadenylation signals were identified in the 3' noncoding sequence of the *DAX-1* gene (underlined). The second of these signals (shown in bold) is used in the only polyadenylated cDNA we isolated (cDNA FA9B, Fig. 1c). The arrow corresponds to the site of polyadenylation. The horizontal arrows correspond to the oligonucleotide primers used to analyse AHC patients. **b**, Amino-acid alignment of the C-terminal ligand binding domain of human *DAX-1* to human *EAR-2*¹³, *Drosophila* protein *seven-up* protein¹⁴, *xenopus* *COUP-TF*¹⁵, human *COUP-TF*¹⁶, human *ARP-1*¹⁷, *Drosophila* *ultraspiracle* protein¹², mouse *RXR γ* ¹¹, human *RXR β* ¹⁰ and human *RXR α* ⁹. The numbers on the left represent the positions of amino-acid residues in the individual receptors. Amino acids identical to *DAX-1* are represented with a line. Similar amino acids are shadowed. The percentages of identity (% id) and similarity (% sim) of each receptor to *DAX-1* are shown on the right. **c**, Amino-acid alignment of the N-terminal repeats of *DAX-1*. Identical amino acids are represented with a line, similar amino acids are shadowed. Identity or similarity restricted to the last two repeats is cross-hatched. **d**, Schematic representation of the *DAX-1* and mouse *RXR γ* proteins. Numbers represent the position of amino-acid residues in the proteins. **METHODS**. All nucleotide sequences were determined using the *fml* DNA sequencing kit (Promega) on double-stranded template. The complete *DAX-1* predicted amino-acid sequence, the N-terminal and the C-terminal portions were used separately as a query to search the non-redundant protein sequence database at the National Center for Biotechnology Information using the BLAST network service⁸. BLAST amino-acid alignments were settled manually.

vector pET-22b¹⁹. The resulting plasmid, pET-DAX was used to generate *DAX-1* in *Escherichia coli*. The protein is readily detected in a western blot analysis using polyclonal anti-DAX-1 antibodies raised against the bacterially generated protein (Fig. 5a). *DAX-1* appears to be abundant in extracts from rat adrenal glands, whereas it is not detectable in others (such as kidney, compare lanes 5 and 6). Importantly, adrenal *DAX-1* precisely comigrates with the protein produced in bacteria.

The *DAX-1* cDNA was also inserted in pSG5, a eukaryotic expression vector containing the SV40 early promoter²⁰. The resulting plasmid was transfected in cultured COS cells and both cytoplasmic and nuclear extracts were prepared. Using the anti-DAX-1 specific antibody we show that most of the *DAX-1* is nuclear. The *DAX-1* expressed after transfection comigrates with the bacterially generated protein and with the natural *DAX-1* protein present in adrenal glands (compare Fig. 5a lanes 2, 5 and 7).

DAX-1 binds DNA

As mentioned above, *DAX-1* has homology to the nuclear hormone receptors. The homology is significant only in the putative ligand-binding domain, whereas the DNA-binding domain is substituted

by a novel domain that contains an amino-acidic repeated motif (Fig. 2d). This structure suggests that the novel amino-terminal portion of *DAX-1* may have DNA-binding properties. To test this hypothesis, we used the bacterially generated *DAX-1* in a gel-shift assay using a RA responsive element (RARE)²¹ as a labelled probe. The results (Fig. 5b) show that *DAX-1* efficiently binds the RARE site. The binding is competed by an excess of the unlabelled RARE oligonucleotide (lanes 5–7), whereas it is not competed by a non-related oligonucleotide (not shown).

We then investigated whether *DAX-1* binding may be influenced by *RAR α* protein, and vice versa. To do so, before DNA-binding, we pre-mixed *DAX-1* and *RAR α* proteins (Fig. 5b, c). We observed binding competition for the same site, but no formation of intermediate mobility complexes.

Transcriptional antagonism

To determine whether the competition observed at the level of DNA binding may have functional consequences at the level of transcriptional activation, we transfected a RARE-CAT²² reporter plasmid with the expression vectors for *RAR α* , *RXR α* and *DAX-1* proteins. Although *RAR α* and *RXR α* show the expected transcriptional activation in the presence of retinoic acid (all-trans) (Fig. 5d, lanes 5, 9 and 16), *DAX-1* does not act as a RA-dependent transcriptional activator under these conditions (lanes 2, 3). However, when cotransfected with *RAR α* and *RXR α* , a reproducible suppression of the transcriptional activation elicited by *RAR α* and/or *RXR α* is observed (lanes 6–8, 10–12 and 17–19). The antagonistic effect of *DAX-1* is observed at equimolar concentrations of transfected expression vector. These results indicate that the *DAX-1* product acts as a down regulator of the activators *RAR α* and *RXR α* .

Discussion

Link between adrenal hypoplasia and hypogonadism. The identification of several patients with microscopic or submicroscopic deletions and clinical signs of a contiguous gene syndrome that may include AHC, HHG, mental retardation, GKD and

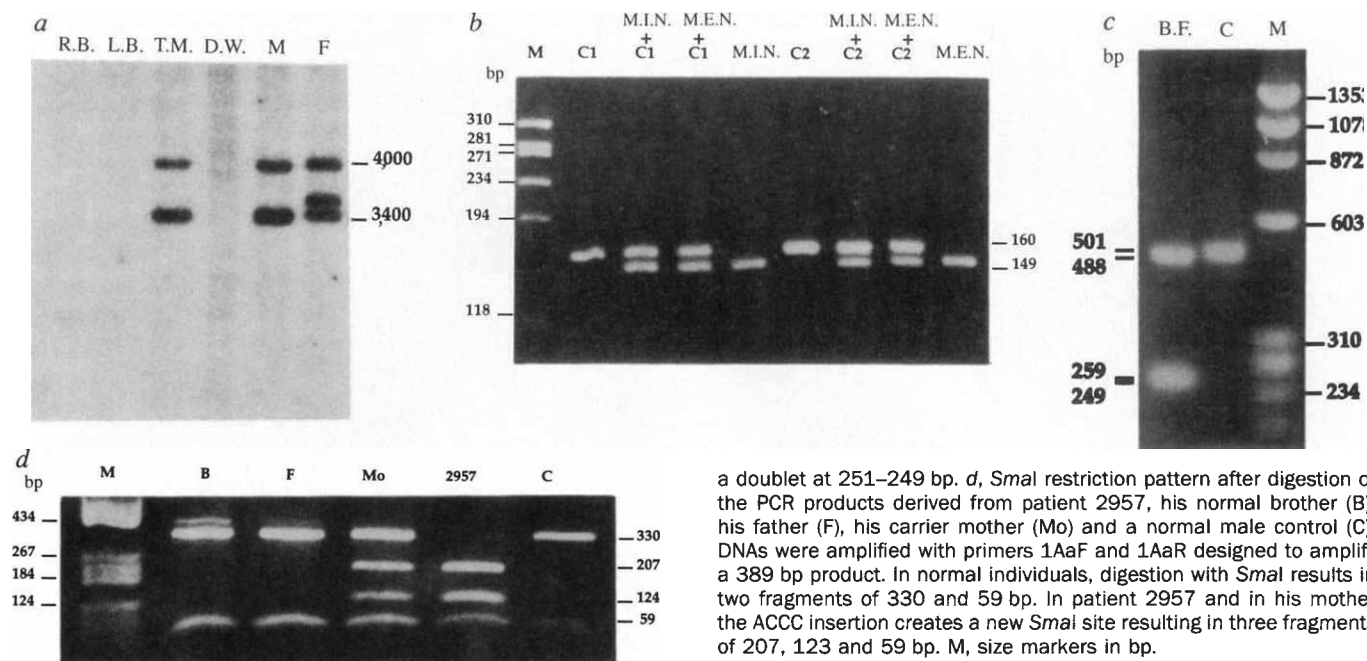


FIG. 3 Deletions and mutations of the *DAX-1* gene in AHC patients. *a*, Southern blot analysis of the *DAX-1* gene in AHC deletion patients. The 10.6 cDNA probe was hybridized to *EcoRI* digestions of previously described patients^{5,6} with deletions in Xp21 and clinical signs of the contiguous gene syndrome AHC, HHG, GKD and/or DMD (see also Fig. 1a). M, Male; F, female controls. The double band in F is due to polymorphism (manuscript in preparation). *b–d*, Confirmation of the mutations detected by direct sequencing in patients. *b*, DNA from brothers M.I.N. and M.E.N. and from two normal male controls (C1 and C2) was amplified with primers 2874 and 2875, designed to amplify a 160 bp product of *DAX-1*. *c*, *MaeI* restriction pattern after digestion of the PCR products derived from patient B.F. and from a normal male control (C). DNAs were amplified with primers 1AF and 1AR. In the control male, digestion with *MaeI* results in a doublet (501 and 488 bp). In patient B.F. a new *MaeI* site is created resulting in one fragment of 488 bp and

a doublet at 251–249 bp. *d*, *SmaI* restriction pattern after digestion of the PCR products derived from patient 2957, his normal brother (B), his father (F), his carrier mother (Mo) and a normal male control (C). DNAs were amplified with primers 1AaF and 1AaR designed to amplify a 389 bp product. In normal individuals, digestion with *SmaI* results in two fragments of 330 and 59 bp. In patient 2957 and in his mother the ACCC insertion creates a new *SmaI* site resulting in three fragments of 207, 123 and 59 bp. M, size markers in bp.

METHODS. Extraction of DNA from peripheral blood, restriction digestions and Southern blotting analysis were all according to standard procedures³⁹. Identification of point mutations in AHC patients was accomplished by direct sequence analysis of PCR-amplified products of genomic DNA isolated from blood samples. The following primer pairs (listed 5' to 3') were used for PCR amplification of patients' *DAX-1* gene: 1AF CACTGGGCAGAACTGGGCTAC; 1AR CTGCAGCATGCTGGGCTCCG; 1AaF CGAAGCGCCCGAGGCGAC; 1AaR CGCTTGATTGTGCTCGTGG; 2875 AGCACAATCAAGCGCAGGC; 2874 AGTACTTGACGAAGCGCAGC. The position of the primers in the *DAX-1* sequence is shown in Fig. 2a. PCR amplifications were in 100 μ l volumes containing 0.1–0.2 μ g of genomic DNA, 50 pmol of each primer, 10 μ l 10 $\times$ reaction buffer supplied by the manufacturer (Boehringer), 200 μ M each dNTPs, 2.5 units *TaqI* DNA polymerase (Boehringer). Samples were denatured for 3 min at 95 $^{\circ}$ C and then amplified for 35 cycles of 45 s at 95 $^{\circ}$ C, 40 s at 68 $^{\circ}$ C, 1 min at 72 $^{\circ}$ C (2 min for the final extension).

DMD, allowed mapping of AHC to Xp21^{4–6}. We have provided evidence that a gene, *DAX-1*, related to the nuclear hormone receptor superfamily, is responsible for the X-linked form of AHC. The *DAX-1* gene maps to Xp21, is expressed in adrenal glands and is deleted in all AHC deletion patients (with one interesting exception) previously used to define the AHC critical interval^{4–6}. Furthermore, we identified mutations that disrupt the integrity of *DAX-1* in three unrelated AHC patients with no detectable deletions or rearrangements in this region. In a more comprehensive screening, we identified point mutations, small insertions or deletions within the coding portion of *DAX-1*, in 12 out of 17 (70%) unrelated AHC patients (F.M. *et al.*, accompanying paper).

Hypogonadotropic hypogonadism is frequently associated with AHC. It was not clear, however, if this association represents a contiguous gene syndrome of is caused by mutations at a single gene. Localization of the HHG locus distal to the AHC locus has been proposed²³. Here we have demonstrated that patients carrying point mutations in *DAX-1* and no detectable deletions or rearrangement in Xp21, can be affected by both AHC and HHG. These data definitively establish that the absence of an intact *DAX-1* is responsible for both AHC and HHG. Further studies will better clarify the role of *DAX-1* in the hypothalamus/pituitary/steroidogenic axis (see also below). ***DAX-1* and gonadal development.** We previously mapped the *DSS* locus to Xp21, adjacent to the AHC locus. Individuals with a double dosage of *DSS* and an intact *SRY* gene develop as phenotypically sex-reversed females. Interestingly, absence of *DSS* is compatible with a male phenotype. We proposed that

DSS has a role in ovarian development and functions as a link between ovary and testis formation⁷.

We show here that *DAX-1*, the gene responsible for AHC, maps within the *DSS* critical interval. The *DAX-1* and *DSS* genes may thus coincide. As the steroidogenic components of the gonads and the adrenal glands have a common embryological origin, it is possible that the *DAX-1* gene has an important role in the development of both organs. Accordingly, disruption of the *Ftz-F1* gene²⁴ (a member of the nuclear hormone receptor superfamily) prevents the development of both the adrenal glands and the gonads²⁵. We failed to detect gross rearrangements in the *DAX-1* gene of thirty 46,XY females. Preliminary *in situ* experiments in developing mouse embryos suggest, however, that *DAX-1* is indeed expressed in the genital ridge (A. Swain *et al.*, manuscript in preparation). The construction of transgenic mice carrying additional copies of the gene will allow to test if *DAX-1* gene dosage does affect gonadal development.

A nuclear protein with a regulatory function. The *DAX-1* gene encodes a protein product of 470 amino acids. The 225 amino acid, C-terminal portion of the *DAX-1* protein shares continuous similarity to the E domain of the nuclear hormone receptor superfamily. The highest homology is observed between *DAX-1* and the RXR^{9–12} and orphan (COUP, ARP-1, EAR-2)^{13–17} receptor subfamilies (see Fig. 2b). The E domain, also called the ligand-binding domain (LBD), has been shown to be important for hormone binding, dimerization and transactivation^{26,27}. Dimerization is obtained through heptad repeats which are a constant feature among nuclear receptors^{10,28}. The amino-acidic sequence of *DAX-1* predicts the presence of a number of heptad

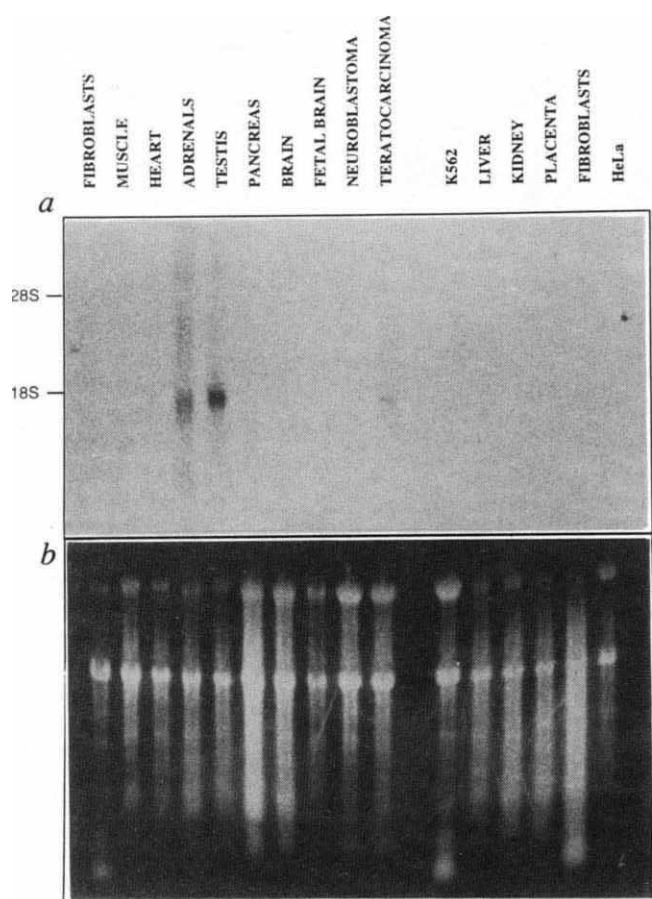
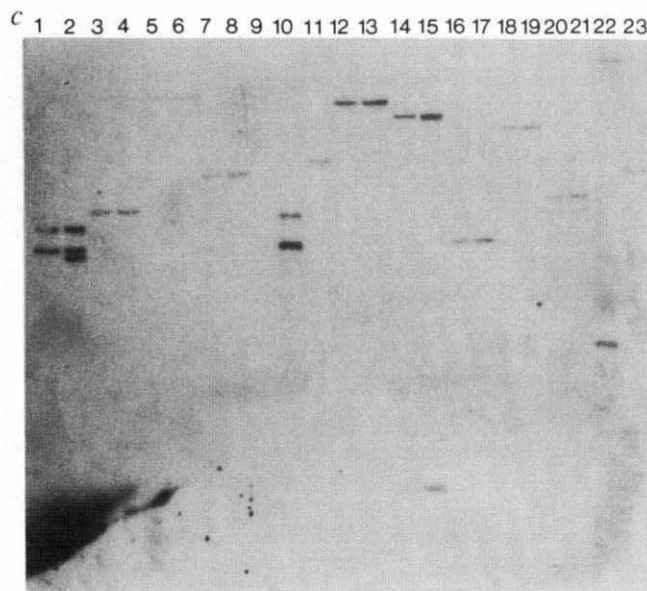


FIG. 4 Pattern of expression and conservation of *DAX-1*. *a*, Northern blot analysis with the *DAX-1* cDNA probe 10.6. Expression was detected in adult testis, adult adrenals and in a teratocarcinoma cell line (N-Tera-2 clone D2⁴³). *b*, Ethidium bromide staining of the RNAs in *a*. *c*, Conservation of *DAX-1* gene across species. Southern blot analysis of DNA obtained from different species, digested with *Eco*RI (lanes



1–13) or *Hind*III (lanes 14–22) and hybridized with the cDNA probe 10.6. Lanes: 1, 2, 14, 15 human; 3, 4, 16, 17 bovine; 5, 6, 18, 19 hamster; 7, 8, 20, 21 mouse; 9 *Drosophila*; 10 *Macaca mulatta*; 23 chicken. Lanes 1, 3, 5, 7, 12, 14, 16, 18, 20 male; lanes 2, 4, 6, 13, 15, 17, 19, 21 female. The double band in lane 2 is due to a polymorphism. **METHODS.** Total RNA was purchased (Clontech) or extracted from cultured cell lines, separated on a 1% agarose gel with 6.1% formaldehyde and blotted onto a nylon membrane (Nytran, Schleicher & Schuell) according to standard procedures³⁹. Restriction digestions and Southern blotting analysis were all according to standard procedures³⁹. Washings were at low stringency (2XSSC at 60 °C) in *c*.

repeats and suggests that *DAX-1* may dimerize. Indeed, the mobility of the complex observed on a RARE suggests that *DAX-1* may bind as a homodimer. Interestingly, *DAX-1* does not heterodimerize with *RAR α* or *RXR α* . The level of homology between *DAX-1* and the other members of the nuclear hormone receptor superfamily, although significant, does not provide a clue about a hypothetical ligand.

The DNA-binding domain (DBD or C domain) is highly conserved among the different types of receptors and is composed of two zinc fingers. In *DAX-1* the C domain is missing and is substituted by a novel N-terminal repetitive structure, organized into four incomplete repetitions of an alanine- and glycine-rich 65–67 amino-acid motif. The repeats, showing 33–70% identity to each other, contain conserved cysteines residues. We have shown here that *DAX-1* is localized mainly in the nucleus and can bind to a RARE element. We have also noticed that the protein can bind to some different recognition sites (not shown), thus suggesting that the *DAX-1* gene could elicit some additional regulatory functions. Therefore the N-terminal structure of *DAX-1*, showing no obvious similarity to previously reported protein sequences, should define a novel DNA-binding domain.

Our transfection experiments demonstrate that the product of the *DAX-1* gene does not act as a RA-dependent or independent activator of the transcription directed by a RARE. Thus, the putative activation domain present in the E region is not functional under the conditions used in this work. By contrast, *DAX-1* inhibits the RA-dependent transcriptional activation elicited

by *RARs* and *RXRs*. As we do not observe formation of *DAX-1*–*RAR* or *DAX-1*–*RXR* heterodimers, repression mediated by *DAX-1* does not seem to require protein–protein association. Instead, as *DAX-1* readily binds RAREs and can compete with *RAR α* binding, it is possible that downregulation is obtained by site occupation. Additional mechanisms cannot, however, be excluded.

The *DAX-1* gene shows a peculiar structural organization. The coding sequence can be divided in two portions: (1) the N-terminal domain, which defines a novel DNA-binding domain, unique among transcription factors; (2) the C-terminal part, with the characteristics of a nuclear hormone receptor ligand-binding domain. Interestingly, no introns in the *DAX-1* gene, separate the ligand-binding domain from the N-terminal repeated structure. *DAX-1* is the first example of a nuclear hormone receptor not containing the canonical zinc-finger DNA-binding domain. A reciprocal situation is found in the *Drosophila* knirps family of nuclear receptors. The three genes that compose the family (*KN1*, *KNRL* and *EGON*), lack the E domain which is substituted by a completely unrelated domain^{29–31}. The E domain is encoded, in the diverse member of the nuclear receptor superfamily, by 1 to 5 exons^{32–36}. Sequence alignment^{27,32} reveals that a unique intron is present in a conserved position in all vertebrate nuclear receptors. Interestingly, the position of this intron is precisely conserved in *DAX-1*. This suggests that the *DAX-1* E domain is derived by duplication of an ancestor nuclear hormone receptor gene. The duplication might have occurred after the arthropod–vertebrate split, as the *Drosophila*

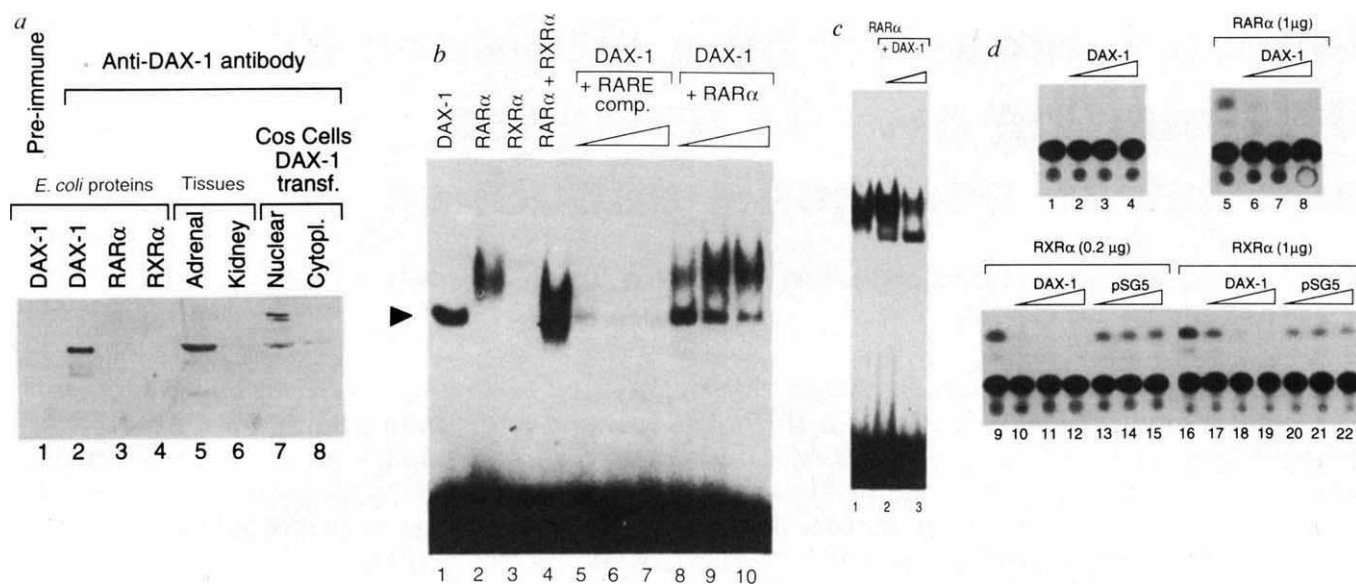


FIG. 5 The DAX-1 protein antagonizes RAR- and RXR-mediated transactivation. **a**, Characterization of DAX-1. The DAX-1 cDNA was cloned in pET-22b¹⁹ and the protein expressed in *E. coli*. Polyclonal antibodies were raised against the full-length protein and assayed in a western analysis. Lane 2, DAX-1 protein is readily detected using the specific antibody. Lane 1, no protein is visible with pre-immune serum. Lanes 3 and 4, the RARα and RXRα proteins are not recognized by the anti-DAX-1-specific antibody. Lane 5, DAX-1 is abundant in adrenal extracts; lanes 6, no DAX-1 is detectable in kidney. Lanes 7 and 8, nuclear and cytoplasmic extracts from COS cells transfected with the DAX-1 expression vector reveal that the protein is mostly nuclear. **b**, DAX-1 binds to a RARE site²¹. Bacterially generated DAX-1 (4 μg, lanes 1, 5–10), RARα (2 μg, lanes 2 and 4) and RXRα (2 μg, lanes 3 and 4) proteins were incubated in a DNA-binding reaction with labelled RARE oligonucleotide. Lanes 5–7, competition of DAX-1 binding with increasing amounts of RARE unlabelled oligonucleotide (10, 50 and 100 pmol, respectively). Lanes 8–10, competition of DAX-1 binding by increasing amounts of RARα protein (1, 3 and 5 μg). **c**, Competition of RARα binding to a labelled RARE oligonucleotide by increasing amounts of DAX-1. Bacterially generated RARα protein (2 μg, lanes 1–3) was incubated in a DNA-binding reaction with a labelled RARE oligonucleotide. Lanes 2, 3, competition of RARα binding by increasing amounts of DAX-1 (2, 6 μg). **d**, DAX-1 downregulates RARα- and RXRα-mediated transactivation. Rac65 cells were transfected with the expression vectors²⁰

for DAX-1, mouse RARα and mouse RXRα, together with the RARE-containing reporter (TRE3)-tk-CAT²². Lanes 1–4, DAX-1 does not transactivate RARE-CAT either in the absence (not shown) or in the presence of retinoic acid (10⁻⁷ M). Coexpression of DAX-1 and RARα (lanes 6–8) and RXRα (lanes 10–12 and 17–19) results in a striking decrease in the transactivation elicited by the receptors the repression is observed at various concentrations of the transfected plasmids. There is no effect of the cotransfected pSG5²⁰ (lanes 13–15 and 20–22).

METHODS. Construction of the full-length cDNA: a 1,083 bp *SacI*–*PvuII* genomic fragment was ligated to a 674 bp *PvuII*–*EcoRI* fragment of cDNA 1.10 and cloned into plasmid BlueScript. A 1,522 bp *NcoI*–*EcoRI* fragment (from the ATG to the polyadenylation site, nucleotides 235–1,757 in Fig. 2a) was then subcloned into the pET-22b vector. Production of the DAX-1 protein in *E. coli* was obtained using the pET22b vector as described¹⁹. Purification of bacterial proteins, preparation of tissue extracts and western blot analysis were as described⁴⁴. The RARE oligonucleotide used in the assays is: 5'GGGTAGGGTTCACCGAAAGTTCACCTCG-3'²¹. Gel shift assays were as described⁴⁵. Transfections in Rac65 cells contained 2 μg of the (TRE3)-tk-CAT reporter and 200 ng (lanes 2, 6, 10, 17), 1 μg (lanes 3, 7, 11, 18) or 2 μg (lanes 4, 8, 12, 19) of the DAX-1 expression vector. Analogous concentrations were used for the pSG5 control. The total plasmid DNA transfected was 10 μg per 10 cm plate. Calcium phosphate coprecipitation was as described⁴⁵.

members of the nuclear hormone receptor family do not have an intron in this conserved position^{27,32} □

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In vivo repression by a site-specific DNA-binding protein designed against an oncogenic sequence

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A DNA-binding peptide comprising three zinc-fingers has been engineered to bind specifically to a unique nine-base-pair region of a *BCR-ABL* fusion oncogene in preference to the parent genomic sequences. Binding to the target oncogene in chromosomal DNA is possible in transformed cells in culture, and results in blockage of transcription. Consequently, murine cells rendered independent of growth factors by the action of the oncogene revert to factor dependence upon transient transfection with a vector expressing the peptide.

THE incorporation of proteins designed to recognize and bind specific DNA sequences into chimaeric transcription factors, recombinases and nucleases, for example, should have a wide range of applications. We have shown that zinc-finger mini-domains can discriminate between closely related DNA triplets, and have proposed that they can be linked together to form domains for the specific recognition of longer DNA sequences^{1,2}. One interesting possibility for the use of such protein domains is to target selectively genetic differences in pathogens or transformed cells. Here we report the first such application, in which we have built a protein which recognizes a specific DNA sequence both *in vitro* and *in vivo*.

There is a set of human leukaemias in which a reciprocal chromosomal translocation t(9; 22) (q34; q11) results in a truncated chromosome 22, the Philadelphia chromosome (Ph)³, encoding at the breakpoint a fusion of sequences from the *c-ABL* proto-oncogene⁴ and the *BCR* gene⁵. In chronic myelogenous leukaemia (CML), the breakpoints usually occur in the first intron of the *c-ABL* gene and in the breakpoint cluster region of the *BCR* gene⁶, and give rise to a gene product, p210^{*BCR-ABL*}, of relative molecular mass (M_r) 210,000 (ref. 7). Alternatively, in acute lymphoblastic leukaemia (ALL), the breakpoints usually occur in the first introns of both *BCR* and *c-ABL*⁸, and result in a p190^{*BCR-ABL*} gene product (Fig. 1)⁹. Facsimiles of these rearranged genes act as dominant transforming oncogenes in cell culture¹⁰ and transgenic mice¹¹. Like their genomic counterparts, the complementary DNAs bear a unique nucleotide sequence at the fusion point of the *BCR* and *c-ABL* genes, which can be recognized at the DNA level by a site-specific DNA-binding protein. We have designed such a protein to recognize the unique fusion site in the p190^{*BCR-ABL*} cDNA. Our experiments are repeated on p210^{*BCR-ABL*} cDNA which serves as a control. The exon fusions found in these cDNAs are obviously distinct from the breakpoints in the spontaneous genomic translocations, which occur in introns and are thought to be variable among patients. Hence although the design of peptides able to bind oncogenes has implications for cancer research, our primary aim here is to prove the principle of protein design, and to assess the

feasibility of *in vivo* binding to chromosomal DNA in available model systems.

The DNA-binding proteins we create are composed of classical zinc-finger motifs¹²⁻¹⁴. These small motifs are ideal natural building blocks for *de novo* protein design because they function as independent modules¹⁵, but can be connected by a well known linker¹⁶ to allow recognition of long, asymmetric DNA sequences. Lately it has been possible to isolate zinc-fingers that bind to given DNA triplets by selection from phage display libraries of randomized zinc-fingers^{1,17,18}. The specificity of selected fingers is checked by a second selection technique called the 'binding-site signature', in which these fingers are used to screen libraries of randomized oligonucleotide-binding sites, thus identifying fingers that can specify a unique base triplet². From these and other studies¹⁹, elements of a recognition code have emerged which relate the amino-acid sequence of zinc-fingers to their cognate triplet.

The strategy we use in creating DNA-binding proteins combines phage display selection and rational design based on the available recognition rules. A 9-base-pair (bp) target sequence (GCA, GAA, GCC) for a three-zinc-finger peptide was chosen which spanned the fusion point of the p190^{*BCR-ABL*} cDNA⁸. The three triplets forming this binding site were each used to screen a zinc-finger phage library over three rounds as described¹. The selected fingers were then analysed by binding-site signatures to reveal their preferred triplet, and mutations to improve specificity were made to the finger selected for binding to GCA (Fig. 2). A phage display mini-library of putative *BCR-ABL*-binding three-finger proteins was cloned in fd phage, comprising six possible combinations of the six selected or designed fingers (1A, 1B; 2A; 3A, 3B and 3C) linked in the appropriate order. The mini-library was screened once with an oligonucleotide containing the 9 bp *BCR-ABL* target sequence, to select for tight binding clones over weak binders and background vector phage. Because the library was small, we did not include competitor DNA sequences for homologous regions of the genomic *BCR* and *c-ABL* genes, but instead checked the selected clones for their ability to discriminate. We found that although all the selected clones were

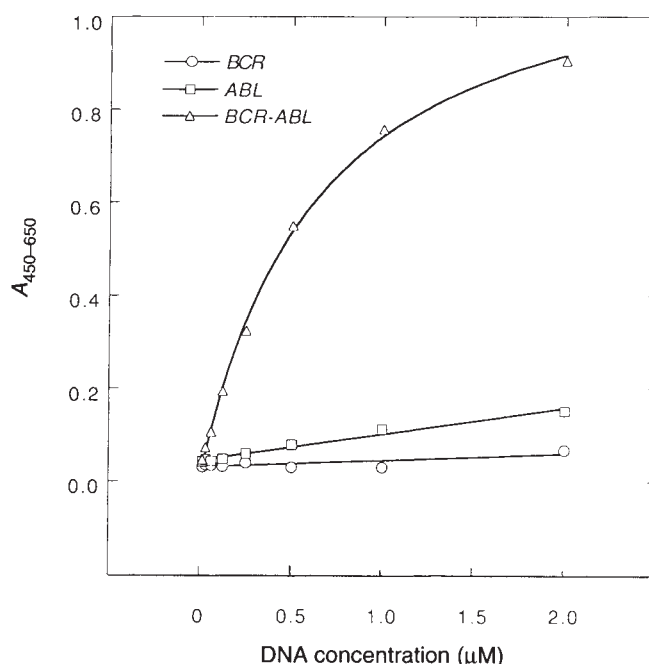
FIG. 1 Nucleotide sequences of the fusion point between *BCR* and *ABL* sequences in p190 cDNA, and of the corresponding exon boundaries in the *BCR* and *c-ABL* genes. Exon sequences are written in capital letters and introns in lower-case letters. Top, p190^{*BCR-ABL*} cDNA; middle, *BCR* genomic sequence at junction of exon 1 and intron 1; bottom, *ABL* genomic sequence at junction of intron 1 and exon 2 (ref. 8). The 9-bp target sequence in the p190^{*BCR-ABL*} cDNA is underlined, as are the homologous sequences in genomic *BCR* and *c-ABL*.

<i>BCR</i> - <i>ABL</i>	TTC CAT GGA GAC <u>GCA G AA GCC</u> CTT CAG CGG CCA
<i>BCR</i>	TTC CAT GGA GAC <u>GCA G</u> gt gag ttc ctc acg cca
<i>ABL</i>	ccc ctt tct ctt <u>cca g</u> AA GCC CTT CAG CGG CCA

FIG. 2 Amino-acid sequences of zinc-fingers used in constructing the mini-library of putative *BCR-ABL* binders. Regions of secondary structure are underlined below the list; residue positions are given above, relative to the first position of the α -helix (position 1). Zinc-finger phages were selected from a library of 2.6×10^6 variants using three DNA-binding sites, each containing one of the triplets GCC, GAA or GCA¹. Binding-site signatures (data not shown) indicate that fingers 1A and 1B specify the triplet GCC, finger 2A specifies GAA, and the fingers selected using the triplet GCA all prefer binding to GCT (ref. 2). These include finger 3A, the specificity of which we believed, on the basis of recognition rules, could be changed by a point mutation. Finger 3B, based on the selected finger 3A, but in which Gln at helical position +2 was altered to Ala, should be specific for GCA. Finger 3C is an alternative version of finger 3A, in which the recognition of C is mediated by Asp +3 rather than by Thr +3.

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FIG. 3 Discrimination in the binding of the anti-*BCR-ABL* peptide to its p190^{*BCR-ABL*} target site and to like regions of genomic *BCR* and *c-ABL*. The graph shows binding (measured as an absorbance between 450 and 650 nm) at various DNA concentrations. Binding reactions and complex detection by enzyme immunoassay were performed as described², and a full curve analysis was used in calculations of the K_d (ref. 16). The DNAs used were oligonucleotides spanning 9 bp either side of the fusion point in the cDNA or the exon boundaries. The anti-*BCR-ABL* peptide binds to its intended target site with $K_d = 6.2 \pm 0.4 \times 10^{-7}$ M, and is able to discriminate against genomic *BCR* and *c-ABL* sequences, although the latter differs by only one base pair in the bound 9-bp region.



able to bind the *BCR-ABL* target sequence and to discriminate between this and the genomic *BCR* sequence, only a subset could discriminate against the *c-ABL* sequence which, at the junction between intron 1 and exon 2, has an 8/9 bp homology to the *BCR-ABL* target sequence⁸. Sequencing of the discriminating clones revealed two types of selected peptide, one with the composition 1A-2A-3B and the other 1B-2A-3B. Thus both peptides carried the third finger (3B) which was specifically designed against the triplet GCA, but peptide 1A-2A-3B was able to bind to the *BCR-ABL* target sequence with higher affinity than was peptide 1B-2A-3B.

The peptide 1A-2A-3B, which we will refer to as the anti-*BCR-ABL* peptide, was used in further experiments. The anti-*BCR-ABL* peptide has an apparent equilibrium dissociation constant (K_d) of $6.2 \pm 0.4 \times 10^{-7}$ M for the p190^{*BCR-ABL*} cDNA sequence *in vitro*, and discriminates against the similar sequences found in genomic *BCR* and *c-ABL* DNA by factors greater than an order of magnitude (Fig. 3). The measured dissociation constant is higher than that of three-finger peptides from naturally occurring proteins such as Sp1 (ref. 20) or Zif268 (ref. 21), which have K_d values in the range of 10^{-9} M, but rather is comparable to that of the two fingers from the *tramtrack* (*ttk*) protein²². However, the affinity of the anti-*BCR-ABL* peptide

could be refined if necessary by site-directed mutations or by 'affinity maturation' of a phage display library²³.

Having established DNA discrimination *in vitro*, we wished to test whether the anti-*BCR-ABL* peptide was capable of site-specific DNA binding *in vivo*. The peptide was fused to the VP16 activation domain from herpes simplex virus²⁴ and used in transient transfection assays (Fig. 4) to drive production of a chloramphenicol acetyl transferase (CAT) reporter gene from a binding site upstream of the TATA box²⁵. A thirtyfold increase in CAT activity was observed in cells cotransfected with reporter plasmid bearing copies of either the *BCR* or *c-ABL* semihomologous sequences. The selective stimulation of transcription indicates convincingly that highly site-specific DNA binding can occur *in vivo*. But although transient transfections assay binding to plasmid DNA, the true target site for this and most other DNA-binding proteins is in genomic DNA. This might well present significant problems, not least because this DNA is physically separated from the cytosol by the nuclear membrane, but also because it may be packaged within chromatin.

To study whether genomic targeting is possible, we made a construct in which the anti-*BCR-ABL* peptide was flanked at

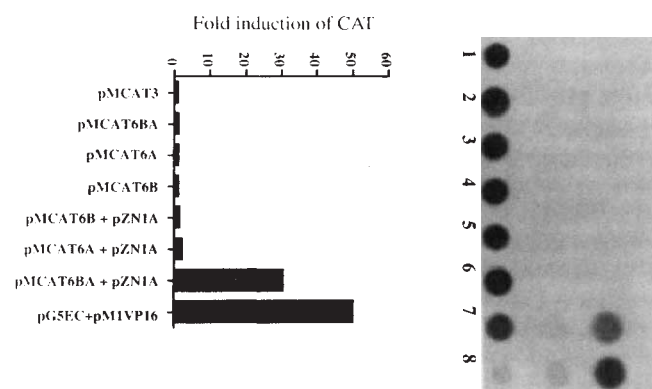


FIG. 4 Transactivation reporter assays using the anti-BCR-ABL peptide fused to the VP16 activation domain. C3H10T1/2 cells were transiently cotransfected with a CAT reporter plasmid and an anti-BCR-ABL/VP16 expression vector (pZNI1A). The top panel shows the results of thin-layer chromatography of samples from different transfections, in which the fold induction of CAT activity relative to a sample in which reporter alone was transfected (lane 1) is plotted on a histogram below. A specific (30-fold) increase in CAT activity was observed in cells cotransfected with reporter plasmid bearing copies of the p190^{BCR-ABL} cDNA target site, indicating binding *in vivo*. The particular constructs used in different transfections are noted below the histogram.

METHODS. Reporter plasmids pMCAT6BA, pMCAT6A and pMCAT6B, were constructed by inserting 6 copies of the p190^{BCR-ABL} target site (CGCAGAAGCC), the *c-ABL* second exon-intron junction sequence (TCCAGAAGCC), or the *BCR* first exon-intron junction sequence (CGCAGGTGAG), respectively, into pMCAT3 (ref. 33). The anti-BCR-ABL/VP16 expression vector was generated by inserting the in-frame fusion between the activation domain of herpes simplex virus VP16 (ref. 24) and the zinc-finger peptide in the pEF-BOS vector³⁴. C3H10T1/2 cells were transiently cotransfected with 10 µg reporter plasmid and 10 µg expression vector. RSVL³⁵, which contains the Rous sarcoma virus long terminal repeat linked to luciferase, was used as an internal control to normalize for differences in transfection efficiency. Cells were transfected by the calcium phosphate precipitation method and CAT was assayed as described³⁶. Plasmid pG5EC, which has five consensus 17-mer GAL4-binding sites upstream from the minimal promoter of the adenovirus E1b TATA box, and pM1VP16 vector, which encodes an in-frame fusion between the DNA-binding domain of GAL4 and the activation domain of herpes simplex virus VP16, were used as a positive control³⁷.

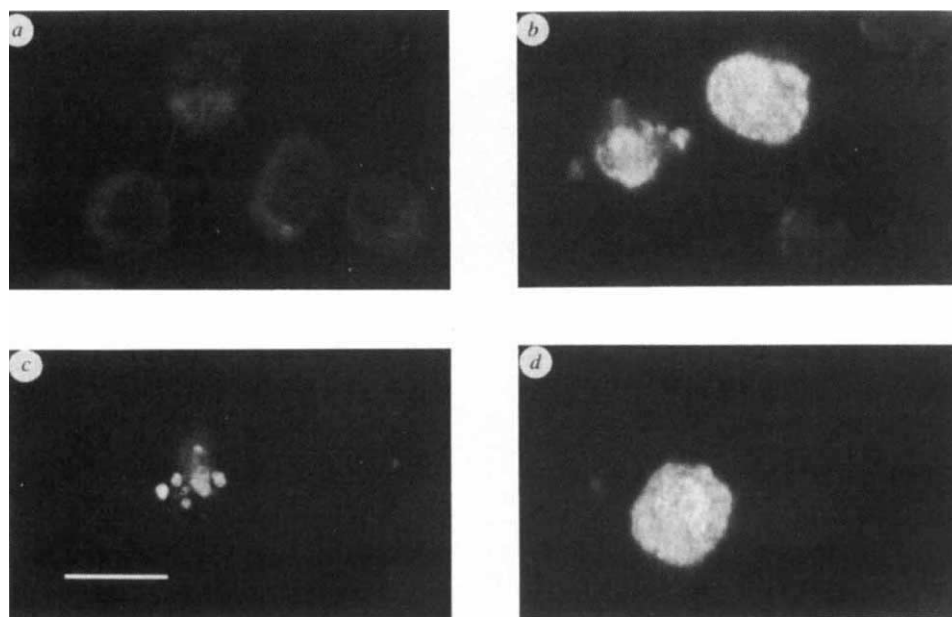


FIG. 5 Immunofluorescence microscopy in the absence of IL-3 of Ba/F3 + p190 and Ba/F3 + p210 cells transiently transfected with the anti-BCR-ABL expression vector and stained with the 9E10 antibody. The images show untransfected cells (a), and transfected cells in which the anti-BCR-ABL peptide is localized in the nucleus (b, c and d). 24 h after IL-3 withdrawal, transfected Ba/F3 + p190 cells show chromatin condensation and nuclear fragmentation into small apoptotic bodies (seen in various stages in b and in c, where the effect is more pronounced). In contrast, neither untransfected Ba/F3 + p190 cells (a) nor transfected Ba/F3 + p210 cells (d) show apoptosis. In b all three types of Ba/F3 + p190 cell are displayed: an untransfected cell (top right); a transfected but still intact cell (middle); and a transfected cell showing nuclear fragmentation (left).

METHODS. The anti-BCR-ABL expression vector was generated in the pEF-BOS vector³⁴, including an 11-amino-acid c-Myc epitope tag (EQKLISEEDLN) at the C-terminal end, recognizable by the 9E10

antibody²⁷ and the nuclear localization signal (PKKKRKV) of the large T antigen of SV40 virus²⁶ at the N-terminal end. Three glycine residues were introduced downstream of the nuclear localization signal as a spacer to ensure exposure of the nuclear leader from the folded molecule Ba/F3 cells were transfected with 25 µg of the anti-BCR-ABL expression construct tagged with the 9E10 c-Myc epitope as described³⁸. Protein production was analysed 48 h later by immunofluorescence-labelling in the presence of IL-3, and also 24 h after subsequent withdrawal of IL-3. Cells were fixed in 4% (w/v) paraformaldehyde for 15 min, washed in PBS and permeabilized in methanol for 2 min. After blocking in 10% fetal calf serum in PBS for 30 min, the mouse 9E10 antibody was added. After 30-min incubation at room temperature, a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (SIGMA) was added and incubated for a further 30 min. Fluorescent cells were visualized using a confocal scanning microscope (magnification, ×90).

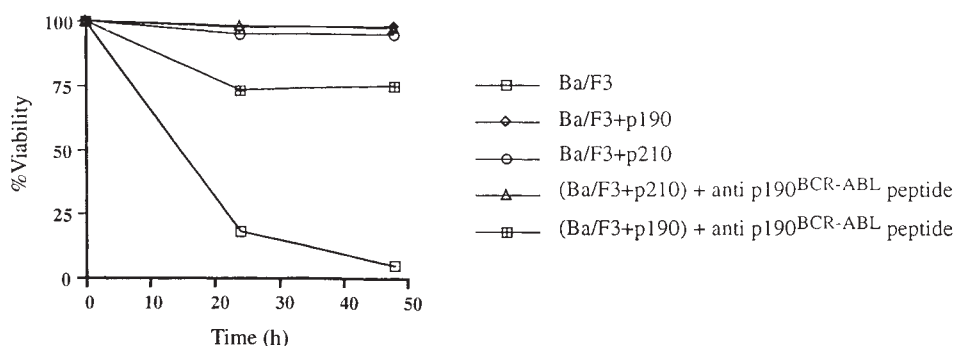


FIG. 6 Viability in the absence of IL-3 of transformed Ba/F3 cells transiently transfected with a vector expressing anti-BCR-ABL peptide. The Ba/F3 cell line is dependent on IL-3 for growth, but becomes IL-3-independent when stably transformed by p190^{BCR-ABL} or p210^{BCR-ABL} cDNA (ref. 10, and I.S.G. *et al.*, manuscript in preparation). A proportion of Ba/F3 + p190 cells corresponding to the proportion transfected with the anti-BCR-ABL expression vector (Fig. 5) revert to IL-3 dependence, but similarly transfected Ba/F3 + p210 cells are unaffected.

the amino terminus with the nuclear localization signal from the large T antigen of SV40 virus²⁶, and at the carboxy terminus with an 11-amino-acid c-Myc epitope tag recognizable by the 9E10 antibody²⁷. This construct was used transiently to transfect the interleukin-3 (IL-3)-dependent murine cell line Ba/F3 (ref. 28), or alternatively Ba/F3 + p190 and Ba/F3 + p210 cell lines previously made IL-3-independent by integrated plasmid constructs expressing either p190^{BCR-ABL} or p210^{BCR-ABL}, respectively (I.S.G. *et al.*, manuscript in preparation). Immunofluorescence microscopy was used to visualize cells stained with 9E10 antibody followed by a secondary fluorescent conjugate (Fig. 5). The efficiency of transient transfection of all cell lines, measured as the proportion of immunofluorescent cells in the population, was 15–20%. The peptide is efficiently localized to the nucleus of all such transfected cells. In the presence of IL-3, all cell lines are viable 48 h after transfection. On the other hand, when IL-3 is subsequently withdrawn from cell culture, over 90% of the transfected Ba/F3 + p190 cells become apoptotic within 24 h (Fig. 5b, c). In contrast, all transfected Ba/F3 + p210 cells remain intact (Fig. 5d). The anti-BCR-ABL peptide is therefore able to restore an IL-3 dependence in the Ba/F3 + p190 cells which the oncogene had abolished.

In another set of experiments in which cell viability in tissue culture after IL-3 withdrawal is assayed by Tripan blue exclusion, 20% of Ba/F3 + p190 cells are found to have reverted to factor dependence and die within 24 h, whereas Ba/F3 + p210 cells are unaffected (Fig. 6). Northern blots of total cytoplasmic RNA from transfected Ba/F3 + p190 cells indicated levels of

METHODS. Cell lines Ba/F3, Ba/F3 + p190 and Ba/F3 + p210 were maintained in DMEM medium supplemented with 10% fetal bovine serum. In the case of the Ba/F3 cell line, 10% WEHI-3B-conditioned medium was included as a source of IL-3. After the transfection with the anti-BCR-ABL expression vector, cells (5×10^5 per ml) were washed twice in serum-free medium and cultured in DMEM with 10% fetal bovine serum without WEHI-3B-conditioned medium. Percentage viability was determined by Tripan blue exclusion. Data are expressed as means of triplicate cultures.

p190^{BCR-ABL} messenger RNA reduced by 15–18% relative to untransfected cells. These results correlate well with the proportion of cells that have become transfected and which die in the absence of IL-3 (Figs 5 and 6). The reduction in mRNA level thus indicates a transcriptional block imposed by the sequence-specific binding of the peptide, which would obstruct the path of the polymerase.

Hence a DNA-binding protein designed to recognize a specific DNA sequence *in vitro*, is active *in vivo* where, directed to the nucleus by an appended localization signal, it can bind its target sequence in chromosomal DNA, causing a specific inhibition of transcription. The use of a blocking agent in this case to target intragenic sequences is reminiscent of antisense oligonucleotide- or ribozyme-based approaches to inhibiting the expression of selected genes²⁹. Like antisense oligonucleotides, zinc-finger DNA-binding proteins can be tailored against genes altered by chromosomal translocations or point mutations. Also, like oligonucleotides that can be designed to repress transcription by triple-helix formation in homopurine-homopyrimidine promoters³⁰, DNA-binding proteins can bind to various unique regions outside genes. But by contrast to nucleic acids, proteins can direct gene expression by both up- or down-regulating the initiation of transcription when fused to activation³¹ or repression domains³².

By acting directly on any DNA, and by allowing fusion to a variety of protein effectors, tailored site-specific DNA-binding proteins have the potential to control expression of specific genes and to further the possibility of manipulating the genetic material itself, in medicine and research. □

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Conformation of GroEL-bound α -lactalbumin probed by mass spectrometry

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The conformation of a three-disulphide derivative of bovine α -lactalbumin bound to the molecular chaperone GroEL has been investigated by monitoring directly its hydrogen exchange kinetics using electrospray ionization mass spectrometry. The bound protein is weakly protected from exchange to an extent closely similar to that of an uncomplexed molten globule state of the three-disulphide protein. Binding to GroEL in this system appears to involve relatively disordered partly folded states resembling intermediates formed in the very early stages of kinetic folding of many proteins *in vitro*.

PROTEIN folding in the cell is assisted by a host of helper proteins including the molecular chaperones, which are thought to safeguard newly synthesized proteins from unproductive interactions that may lead to aggregation^{1,2}. On the chaperone-assisted folding pathway, the chaperonins (GroEL and GroES in *Escherichia coli*) are thought to help folding of their substrate proteins to native-like states³. To unravel the molecular details defining the mechanism of action of this important family of proteins, new approaches to the problem of how to determine the conformational properties of a relatively small substrate protein in the presence of the large (M_r 800K) chaperonin oligomer are needed. Here we describe the development of one such approach, shown schematically in Fig. 1, designed to enable information from hydrogen exchange protection, a technique which has revolutionized studies of protein folding *in vitro*^{4,5}, to be obtained from complexes relevant to folding in the cell. We demonstrate that the GroEL–protein substrate complex can be directly monitored by electrospray ionization mass spectrometry (ESI-MS)⁶ and that the GroEL subunits and the ligand can both be visualized in the same spectrum. When combined with hydrogen exchange labelling, this approach allows the study of individual components of the complex, which retain the history of the complexed state by virtue of their deuterium content, and circumvents the need to quench exchange and dissociate the complex before measurement as in more classical approaches^{4,5,7}. By observing directly and in ‘real time’ the hydrogen exchange properties of a partially folded protein bound to the GroEL oligomer, we show the power of ESI-MS to measure selectively protection of individual components in a mixture of species and in proteins that are well beyond the molecular mass range amenable to detailed nuclear magnetic resonance (NMR) analysis. In addition, the ESI-MS method has the unique property that it detects populations of protein molecules distinguished by different numbers of protected amides⁸. We use this information to examine the distribution of conformational states of the protein substrate bound to GroEL. The results of these experiments add a new dimension to our understanding of the molecular details of substrate recognition by GroEL, which hitherto were

restricted to studies of small peptide substrates in rapid exchange with the chaperonin⁹, of the released polypeptide⁷, and of low-resolution electron microscopy images of the bound substrate in the central cavity of GroEL^{10–13}.

GroEL recognizes many different substrates

The homotetradecameric chaperonin GroEL interacts with a wide variety of protein substrates¹. Among the structural features proposed to be recognized by GroEL are α -helices⁹, potential β -sheet regions¹⁴, hydrophobic patches², certain amino-acid side chains¹⁵ and some, but not all, molten globule states^{16–19}. Of the large number of molten globule states described so far²⁰, some of the best characterized are those formed by the α -lactalbumins²¹. These may be induced under a variety of conditions including removal of the single Ca^{2+} ion¹⁹, at low pH²¹ and by reduction of one or more of the four disulphide bridges²². In its native state α -lactalbumin contains both α -helical and β -sheet structures largely segregated into two domains^{23,24}. Although the molten globule states of this and other proteins differ in detail, ranging from highly ordered to largely disordered states²⁰, they all contain substantial secondary structure, but vary in the extent of their tertiary interactions^{21,25}, and are protected from hydrogen exchange to different degrees^{26,27}. In the acid-induced molten globule state, amides in the α -domain are the most highly protected from hydrogen exchange^{25,27}. In this respect the molten globule state closely resembles transient intermediates populated during the kinetic folding pathway of the homologous protein, hen lysozyme, which have been extensively characterized by a wide range of techniques²⁸, including hydrogen exchange labelling detected by NMR²⁹ and ESI-MS⁶. Thus, α -lactalbumin is an excellent model system to study the mode of substrate binding by GroEL. Here we focus on a disulphide-shuffled, three-disulphide partially folded derivative of bovine α -lactalbumin (BLA), termed [3SS], which is formed by removal of the single Ca^{2+} ion, selective reduction of the Cys 6–Cys 120 disulphide bond and rearrangement of the three remaining disulphide bonds²². By contrast with native holo-BLA and the molten globule state of apo-BLA with four disulphide bonds^{18,19}, a large proportion of the equilibrium mixture of disulphide isomers in [3SS] has recently been shown to form a stable complex with GroEL (referred to as the GroEL:

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FIG. 1 Schematic diagram of the experiment designed to monitor hydrogen exchange in the GroEL:[3SS] BLA complex by ESI-MS. Before formation of the complex, all exchangeable sites in α -lactalbumin were deuterated by incubation of the apo-protein in D_2O (see legend to Fig. 2 for experimental details). Hydrogen exchange is initiated by a 10-fold dilution of the complex into H_2O , pH 5.0 at 4 °C. Dissociation of the GroEL into monomers in the gas phase releases the bound polypeptide ligand, while maintaining the protection in the substrate protein (see legend to Fig. 2 for details). In this manner, the rate of hydrogen exchange of [3SS] α -lactalbumin (and GroEL) in the complex can be measured directly as a change in their masses as a function of the incubation time in H_2O by ESI-MS.

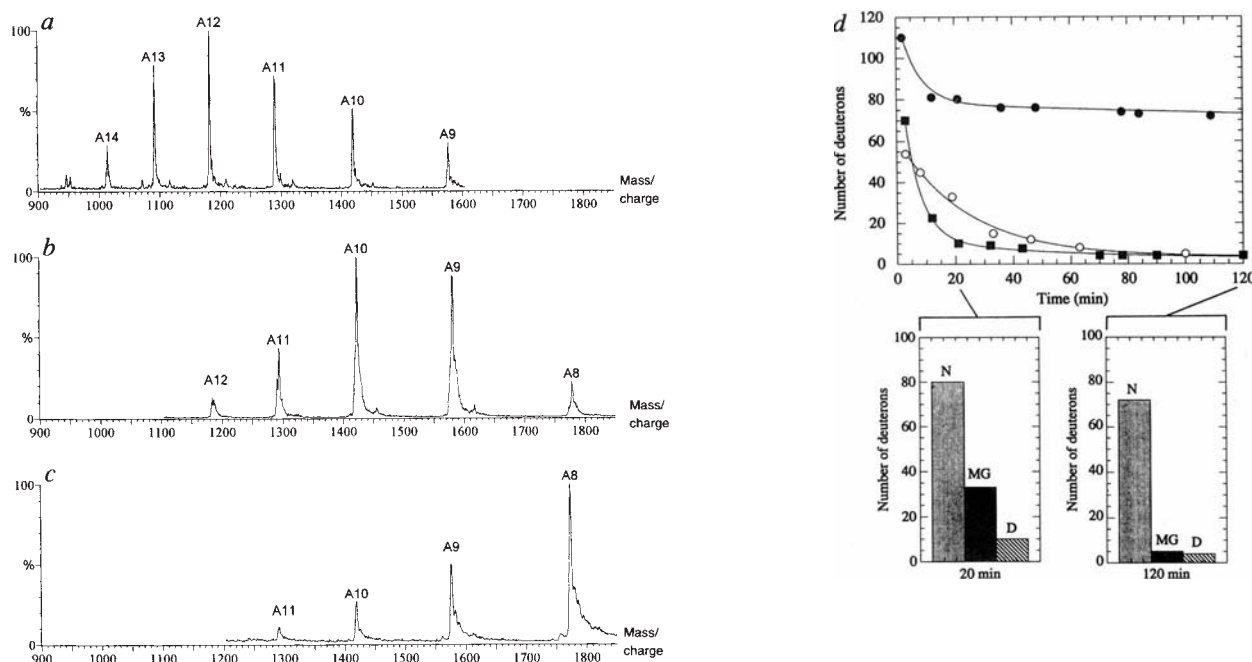
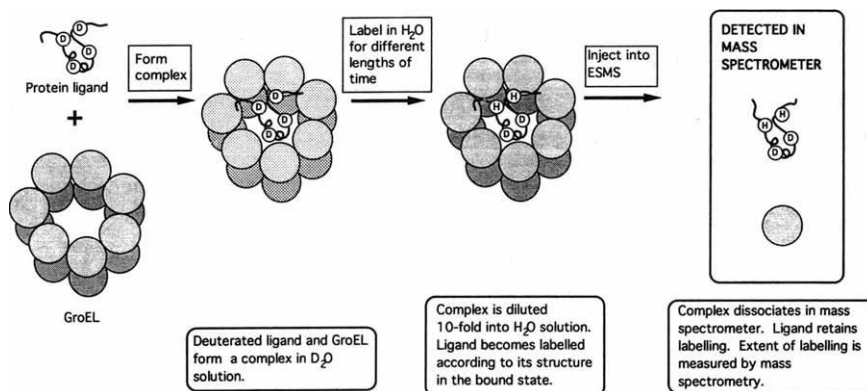


FIG. 2 ESI mass spectra of BLA obtained from 50% (v/v) acetonitrile, 1% (v/v) formic acid (a) water pH 5.0 (b) and water pH 2.0 (c). d, Hydrogen exchange kinetics of different conformational states of hen egg-white lysozyme and α -lactalbumin in water at 20 °C, monitored by ESI-MS. ●, Native hen lysozyme (pH 5.0); ○, BLA (molten globule state at pH 2.0); ■, CM^{6,127}-lysozyme (denatured state at pH 2.0). Measurements were taken over a total exchange time of 180 min but for clarity only the first 120 min are shown here. Histograms are shown to emphasize the numbers of deuterons remaining in the different states after 20 and 120 min. N, Native state of hen lysozyme; MG, molten globule state of BLA at pH 2.0; D, denatured state of CM^{6,127}-lysozyme at pH 2.0. The mass of each sample was determined to within an average error of ± 1.2 mass units. The individual errors are too small to show on the figure.

METHODS. In all experiments ultrapure water (ELGA maxima system) and D_2O (Fluorochem) were adjusted to the required pH by the addition of formic acid (HPLC grade) and ammonia (AnalaR). BLA and hen lysozyme were purchased from Sigma and were dialysed extensively against ultrapure water before use. CM^{6,127}-lysozyme was prepared as described (refs 36, 50). BLA was deuterated at all exchangeable sites by overnight incubation of the apo-protein in D_2O , pH 5.0, 20 °C. Lysozyme was deuterated at all exchangeable sites by incubation of the protein in D_2O for 10 min at 80 °C at pH 4.0. The protein was then freeze-dried, redissolved in D_2O pH 5.0 and washed five times on a Centricon-10 (Amicon) with D_2O pH 5.0. Exchangeable sites in CM^{6,127}-lysozyme were deuterated by incubation of the protein in D_2O , pH 4.0 at 60 °C for 10 min. To initiate the exchange reaction, each protein

sample ($1.5 \text{ nmol } \mu\text{l}^{-1}$) was diluted 100-fold into H_2O at pH 5.0 or pH 2.0 at 20 °C. Mass spectra were recorded on a VG Platform Electrospray Mass Spectrometer (Fisons Instruments). Samples were introduced through a rheodyne injector and were pumped at a flow rate of $10 \mu\text{l min}^{-1}$ into the electrospray interface with a fluid delivery module. The conditions of the electrospray were carefully controlled to study hydrogen exchange in labile states. This included pre-cooling the delivery solvent (water at pH 5.0 or 2.0) in an ice/salt bath. In each experiment the sample was also rapidly pre-cooled to 4 °C before injection into the electrospray interface. The latter was equilibrated overnight with water at either pH 2.0 or pH 5.0 depending on the solution conditions in the exchange experiment. The source heaters were set at 20 °C throughout. The nebulizer gas was also cooled by passing through a copper coil immersed in an ice/salt bath placed immediately before the electrospray interface. The mass spectrometer was calibrated using hen egg-white lysozyme and spectra were obtained within the mass/charge range $1,500 \pm 400$. The M_r of apo-BLA was determined to be 14,178.06 (for the neutral molecule) by accurate mass measurement on a VG Autospec (Fisons Instruments), using hen lysozyme as an internal standard. This result is in full agreement with the amino-acid sequence of the protein^{32,33}. From this, the M_r of the holo-form was calculated to be 14,216, corresponding to addition of one calcium-ion and removal of two protons. Each mass determination was done at least twice. Data were fitted to the sum of two exponentials using the program Kaleidagraph (Abelbeck Software), but because of the difficulty in fitting such complex kinetics to a limited sum of exponential terms, fits are used here for illustrative purposes only.

[3SS] BLA complex), such that one molecule of α -lactalbumin is bound, on average, to each GroEL oligomer¹⁸.

Hydrogen exchange in different states

The ESI mass spectrum of BLA obtained under standard electrospray conditions⁶ in 50% (v/v) acetonitrile and 1% (v/v) formic acid with a source temperature of 50 °C is shown in Fig. 2a. The relative molecular mass (M_r) of $14,178.04 \pm 0.83$ and the charge state maximum of +12 are consistent with the loss of Ca^{2+} and denaturation of the molecule under these conditions. Although acidic acetonitrile solution is ideal for obtaining spectra of high sensitivity appropriate to the accurate measurement of protein M_r s, this approach precludes the study of proteins in their native states. Although this can be overcome by introducing protein molecules from water in the absence of organic cosolvents, spectra of high mass accuracy under these conditions have only been observed in a few systems^{30,31}. We show in Fig. 2b that good quality spectra of BLA of high mass accuracy can be obtained in the absence of source heating (20 °C) at less acidic pH (5.0) and without added cosolvent. The M_r obtained under these conditions ($14,216.8 \pm 1.4$), represents the Ca^{2+} -bound native state^{32,33}. This demonstrates that the protein retains its native structure under these conditions at least until its evaporation in the mass spectrometer. In accord with this, the charge state maximum of this state is significantly different from that of the protein denatured in acetonitrile. The spectrum of the molten globule state of BLA obtained from aqueous solution at pH 2 is shown in Fig. 2c. The M_r ($14,178.8 \pm 0.7$) is again that of the apo protein, in accord with the fact that Ca^{2+} does not bind under these conditions³⁴. Interestingly, the charge state distribution is different from that of the native state and the state obtained from acetonitrile solution. Charge state differences reflecting conformational alterations³⁵ have also been observed in spectra of other molten globule and denatured states (C.V.R. *et al.*, unpublished data) and is likely to reflect the distinct conformational preferences of the partially folded state.

Measurement of the hydrogen exchange kinetics of the different conformational states of proteins by ESI-MS presented a further challenge because, especially for the more labile states, significant exchange can occur during sample introduction into the mass spectrometer. To overcome this the temperature of the nebulizer gas and delivery solvent were reduced and the sample was rapidly pre-cooled before introduction into the mass spectrometer (see legend to Fig. 2 for details). Even under these conditions, high quality spectra were obtained with sufficient sensitivity and reproducibility (on average ± 1.2 mass units) to permit accurate measurement of hydrogen exchange. Using this approach the hydrogen exchange kinetics of the native state of hen lysozyme (pH 5), the molten globule state of BLA (pH 2), and the acid-denatured state (pH 2) of a three-disulphide derivative of lysozyme, in which the Cys 6–Cys 127 disulphide bond has been reduced and the resulting thiols carboxymethylated ($\text{CM}^{6,127}$ -lysozyme)³⁶, were measured in aqueous solution at 20 °C. Substantial differences between the kinetic profiles for the three states were observed (Fig. 2d). The native state of lysozyme is by far the most protected state studied. In agreement with previous data determined by NMR^{37,38}, an average of about 75 deuterons are highly protected from exchange after 2 h under these conditions. By contrast the unfolded state of $\text{CM}^{6,127}$ -lysozyme, which as suggested by NMR methods is extensively denatured under these conditions³⁸, is not protective against hydrogen exchange; the kinetics observed here agree closely with those calculated for a theoretical random structure of this sequence^{39,40}. Even for this unprotected state, the time course of exchange is entirely consistent with similar measurements made by ¹H NMR^{38,41}, demonstrating the success of the ESI-MS approach. The ESI-MS data show that the acid-induced molten globule state of BLA is only weakly protected from hydrogen exchange relative to the native state of lysozyme and that it is more highly protected than the fully denatured state of $\text{CM}^{6,127}$ -

lysozyme (Fig. 2d). The exchange kinetics measured here are also entirely consistent with similar measurements made by NMR^{25,27}.

A unique property of ESI-MS is its ability to monitor distinct populations of protected molecules which, in a hydrogen exchange experiment, possess deuterons which are protected to different extents⁸. In the experiments described here, the nature of the distribution of conformations sampled in the molten globule state contributes to the widths of peaks in the mass spectrum. The peak arising from the +10 charge state in the mass spectrum of the acid-induced molten globule state of BLA throughout the exchange time course is relatively narrow, suggesting that the distribution of conformations populated in this state is much narrower than that represented by a large number of species ranging in their protection from that observed for the native state of lysozyme to the substantially denatured state of $\text{CM}^{6,127}$ -lysozyme. In the native state of hen lysozyme the majority of protected amides have protection factors ranging from 10^3 to 10^8 (ref. 38). By contrast, the number of significantly protected amides in the acid-induced molten globule state is small and their protection factors, like those in the molten globule states of other α -lactalbumins^{25,27,41}, and indeed of other proteins^{26,42} are often less than 10 and rarely exceed values above 50.

Analysis of the GroEL tetradecamer by ESI-MS

The ESI mass spectrum of GroEL obtained under standard ESI-MS conditions in acidic acetonitrile is shown in Fig. 3a. The measured M_r of $57,196.0 \pm 5.5$ is in excellent agreement with the M_r of the GroEL monomer of 57,197.8 calculated from the amino-acid sequence inferred from the revised gene sequence⁴⁴. There is no evidence for higher mass species under these conditions, demonstrating that complete dissociation of the oligomer to the monomeric form had occurred. An important prerequisite for our studies of the GroEL:[3SS] BLA complex was that we could establish conditions whereby GroEL can be maintained in its native tetradecameric conformation in water until its evaporation in the mass spectrometer. Because of the sensitivity of ESI-MS to buffer salts, it was necessary to achieve this in the absence of divalent cations, which are usually present and are

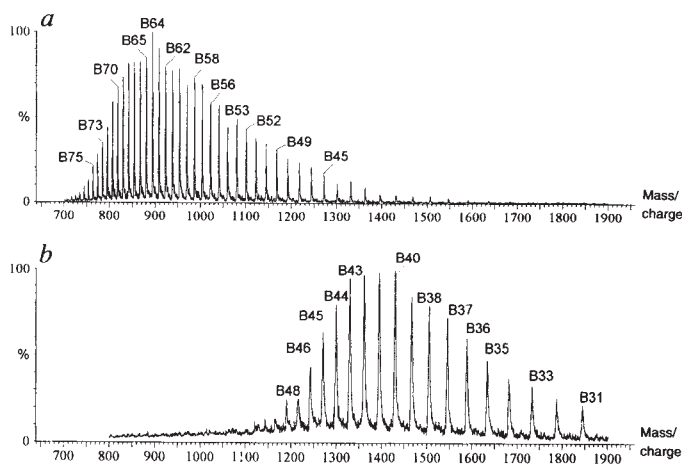


FIG. 3 ESI mass spectra of GroEL obtained from 50% (v/v) acetonitrile, 1% (v/v) formic acid (a) and from water, pH 5.0 (b).

METHODS. The spectrum in a was recorded under standard ESI-MS conditions which include the use of 50% (v/v) acetonitrile and 1% formic acid (v/v) and source heating set to 50 °C. The spectrum obtained from water (b) was recorded using the conditions developed for studying labile states (see legend to Fig. 2). GroEL was overexpressed and purified as described¹⁶, and stored at -80 °C in 20 mM MOPS/KOH pH 7.2 containing 50 mM NaCl, 1 mM DTT, 10% (v/v) glycerol. Before use, the buffer was exchanged for water/formic acid pH 5.0 by repeated cycles of dilution of the sample into this solution and concentration of the diluted protein by ultrafiltration on a Centricon-100.

known to increase the stability of the GroEL tetradecamer⁴⁴. Circular dichroism (CD) experiments in the near and far ultraviolet combined with gel filtration established that the native structure of the chaperonin is maintained in water at pH 5.0 for a period of at least 24 h provided that the temperature is low ($\sim 5^\circ\text{C}$). These conditions, therefore, were chosen for the hydrogen exchange experiments outlined in Fig. 1 and described below.

The ESI mass spectrum of the GroEL tetradecamer introduced into the electrospray interface in water without any organic cosolvents, at pH 5 and 20°C , is shown in Fig. 3b. A dramatic change in the charge state distribution relative to that seen under denaturing conditions is observed; the increased population of lower charge states suggests that the GroEL subunits are more structured under these conditions. Clearly, even under the optimized mass spectral conditions, which had previously been shown to preserve α -lactalbumin in the calcium-bound form, the dominant species observed in the spectrum is the GroEL monomer (M_r 57,204.5 $\pm$ 4.9). This suggests, in agreement with the data of others⁴⁵, that monomerization of GroEL is a relatively facile process. Under some conditions, however, including varying the capillary and skimmer voltage and increasing the protein concentration, small amounts ($<10\%$) of higher oligomers of GroEL can be observed. The ability to dissociate the complex in the mass spectrometer is, however, a prerequisite of our experimental design (Fig. 1) and conditions were chosen to ensure that this occurred.

Hydrogen exchange protection

The ESI mass spectrum of the complex of deuterated [3SS] BLA bound to GroEL recorded 27 min after diluting the complex into H_2O , pH 5.0, 4°C is shown in Fig. 4a. Two distinct series of peaks can be clearly seen in the spectrum, one (labelled B) having a mass corresponding to the GroEL monomer and the other (labelled A) to a partially deuterated apo-BLA species. Integration of the peaks in the transformed spectrum of the complex (Fig. 4b) gave a ratio of 16($\pm$ 4):1, consistent with the 14:1 stoichiometry of GroEL and BLA in the complex. Although different proteins may produce very different response factors in the mass spectrometer, such measurements suggest that at least the majority of the GroEL oligomers in the experiment contain, on average, a single bound BLA molecule. The simplest explanation for the observation of the α -lactalbumin series in the spectrum of the complex is that the ligand has been released from the chaperonin as a consequence of the dissociation of the GroEL oligomer in the gas phase. Indeed, gel filtration experiments confirmed that all of the α -lactalbumin present in the sample before the electrospray was bound to the chaperonin. The dissociation of the complex in the mass spectrometer provides us with a unique opportunity to monitor directly the conformation of a protein substrate bound to the chaperonin by a hydrogen exchange methodology under conditions where protection, even in very labile states, is preserved.

Hydrogen exchange was measured in the GroEL:[3SS] BLA complex following the experimental design outlined in Fig. 1. In

this experiment, [3SS] BLA which had been previously deuterated at all exchangeable sites was bound to the chaperonin in D_2O solution and exchange was initiated by 10-fold dilution of the complex into H_2O , pH 5.0 at 4°C . To relate the hydrogen exchange kinetics of chaperonin-bound [3SS] BLA to known conformational states of the protein, experiments were done under the same conditions with holo-BLA (native state) and with a three-disulphide derivative of BLA in which the Cys 6–Cys 120 disulphide bond has been reduced and the resulting thiol moieties carboxyamidomethylated (apo 3SScam-BLA). This forms a molten globule state under the conditions of our experiments (at pH 5.0, 4°C (M.G. and S.E.R., unpublished data)) as well as at room temperature^{46,47}. Data were also simulated for exchange in a random structure of this sequence under the same conditions (see legend to Fig. 5 for details). Comparison of the kinetic traces in Fig. 5 clearly shows that chaperonin-bound [3SS] BLA is less protected than the native holo-protein and substantially more protected than a random structure. Indeed, the hydrogen exchange profile resembles most closely that of the free molten globule state of apo 3SScam-BLA. Thus, after 20 min at 4°C (Fig. 5b), exchange in a random structure would be complete, whereas about 20 deuterons, on average, are protected from exchange in the free molten globule and in the bound polypeptide after this time.

When exchange was monitored at pH 5 at 20°C , only 6 deuterons were found to be unexchanged in GroEL-bound [3SS] BLA after 23 min (Fig. 6a). By contrast, some 325 deuterons are protected from exchange in each GroEL monomer after this time, representing a remarkable total of 4,550 deuterons in the GroEL oligomer. After 55 min at 20°C , however, a marked change in the spectrum is observed (Fig. 6b). First, the characteristic charge states arising from [3SS] BLA could not be detected in the spectrum and a change in the charge state distribution of GroEL to more highly charged species was observed. In addition, exchange of more than 195 deuterons per GroEL monomer, or 2,730 deuterons in the GroEL oligomer, had occurred. This suggests that dissociation of the protein ligand from GroEL causes major conformational changes in the chaperonin as revealed by the change in the charge state distribution and the loss of large numbers of protected deuterons, and is consistent with recent electron microscopy images of the empty and protein-bound chaperonin¹³. In addition, it shows that in the presence of the 14-fold molar excess of GroEL it was not possible to visualize BLA in the spectrum unless it had been bound to the chaperonin.

The nature of the bound state

The result that amides are protected from hydrogen exchange in [3SS] BLA bound to GroEL suggests that at least some regions of the protein ligand are persistently structured in the bound state. A contribution to protection may also arise as a consequence of the binding of [3SS] BLA to the GroEL central cavity. Although we cannot exclude the possibility that some of the protection arises from binding, the observation that not only the number of protected amides but also that their extent of

FIG. 4 a, ESI mass spectrum of GroEL:[3SS] BLA complex, 27 min after initiating exchange at 4°C in water at pH 5; b, mass-transformed spectrum of the complex.

METHODS. To form the complex, BLA (22.5 μM) deuterated at all exchangeable sites was bound to GroEL (4.5 μM oligomer) by a 10 min incubation at 20°C in a D_2O solution containing 100 mM KCl, 0.5 mM EGTA and 0.5 mM DTT, pH 7.0. The complex was then washed five times with D_2O adjusted to pH 5.0 with formic acid and containing 0.5 mM DTT, by ultrafiltration at 4°C on a centricon-100 to buffer exchange the sample and purify the α -lactalbumin:GroEL complex. The complex was concentrated to 25 μM by a similar ultrafiltration step, stored on ice and used within 24 h. In these spectra, and in all other figures, the charge state series of BLA are labelled A, and those of GroEL, B.

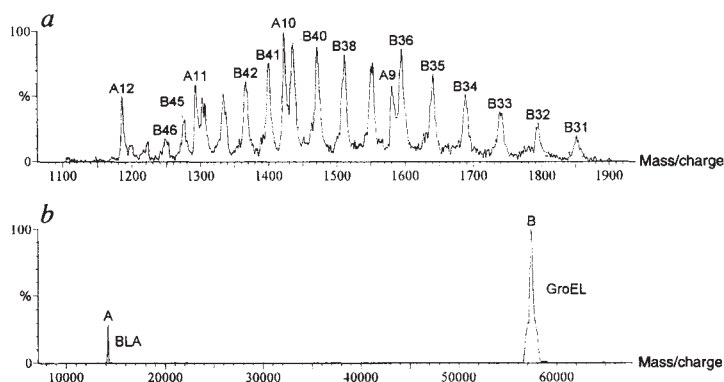
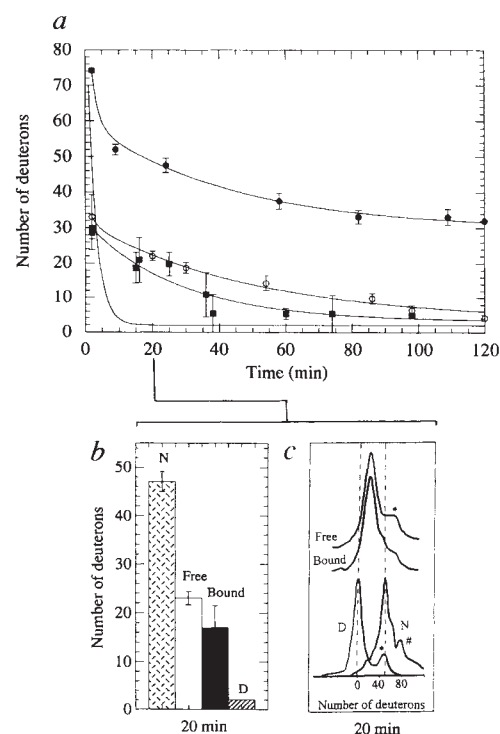


FIG. 5 *a*, Kinetic profiles of hydrogen exchange at pH 5.0, 4 °C, monitored by ESI-MS. ●, Holo-BLA (native state); ○, 3SScam-BLA (molten globule state); ■, [3SS] BLA in a complex with GroEL; line, simulated curve expected for BLA in a completely unstructured conformation. The unbound [3SS] species was not included in this study because it aggregated under these conditions. Data for the exchange profile of completely unstructured BLA were calculated from the amino-acid sequence of the protein, using the recently recalibrated near-neighbour inductive effects on acid, base and water catalysis³⁹, taking into account the fact that exchange is monitored for the deuterated sample in H₂O solution⁴⁰. In addition, the intrinsic rate of exchange of the 97 exchangeable amino-acid side chain deuterons^{32,33}, which would also contribute to the measured mass, especially at early time points, was calculated and included in the simulated curve. The total of 220 exchangeable sites in the complete sequence of BLA and the final D₂O content of 10% (v/v), therefore, result in a mass of BLA 22 units larger than that of the fully protonated apo-form. Data for each of the states studied were corrected to 1% residual D₂O content, to allow comparison with the results in Fig. 2*d*. Measurements were done over a total exchange time of 180 min but for clarity only the first 120 min are shown here. It is intriguing to note that the degree of protection of amides in [3SS] BLA bound to GroEL is slightly, but significantly, lower than that observed in the free partially folded state of 3SScam-BLA over virtually the entire time course. Given that the species examined in the complex is a mixture of three disulphide isomers of BLA the molecular origin of this effect is difficult to interpret in detail. It suggests, however, that the molten globule state of 3SScam-BLA (which has three native disulphide bonds) is more persistently structured than the bound species. *b*, Histogram showing the number of deuterons in the native state of holo-BLA (N), the free molten globule state of 3SScam-BLA (Free), the state of [3SS] BLA bound to GroEL (Bound), and in a simulated random coil structure of BLA (D), 20 min after the initiation of exchange. Error bars represent one s.d. from the average mass calculated for four charge states for each species. *c*, Representative charge states (+12) for holo-BLA (N), the molten globule state of apo 3SScam-BLA (Free), the chaperone-bound state of [3SS] BLA (Bound) 20 min after the initiation of exchange, and a fully exchanged state of apo 3SScam-BLA obtained by heating the sample to 50 °C for 10 min in 90% (v/v) H₂O, 10% (v/v) D₂O before acquisition of the spectrum under conditions identical to those used for the other samples (D). For consistency the mass scale has been normalized to 1% residual D₂O. The +12 charge state in the spectrum of holo-BLA is very weak (Fig. 2*a*). For this reason the peak shown here is the +10 charge state. The peak width of this state has been adjusted to that of a +12 charge state for illustrative purposes. The absolute peak width (width at half height multiplied by the charge) was reproducible for each of the charge states measured for each protein and between replicate samples. The small peak to high mass in the spectrum of holo-BLA (*) arises from the presence of a second Ca²⁺ ion, which is known to bind weakly to the native protein⁵¹. In the spectrum of 3SScam-BLA an additional carboxyamidomethyl moiety can be seen in a small population of molecules (#).



protection resembles closely that of the molten globule state of 3SScam-BLA in free solution suggests that the contribution to protection from binding is small and that the bound polypeptide adopts a structure akin to this well characterized molten globule state. The demonstration that the bound polypeptide is more weakly protected than the native protein is consistent with the conclusions of ref. 7 that the protection factors of amides in cyclophilin bound to GroEL are less than 10^3 . However, given that in typical molten globule states few amides have protection factors exceeding values around 10 (refs 27, 41, 42), the importance of our approach lies in its ability to examine directly and in a quantitative manner small numbers of marginally protected deuterons. Our approach also avoids the necessity that the deuterons monitored must also be protected in the native protein which restricts the analysis of hydrogen exchange by pulse labelling and NMR. In addition, it has the unique advantage that populations of molecules that are not protected from hydrogen exchange can also be directly and quantitatively measured⁸. Our results, therefore, rule out the possibility that the substrate becomes completely unfolded on binding (assuming that the central cavity of GroEL is solvent filled and that the contribution to protection by binding is small) and show clearly and in full accord with earlier predictions⁴⁸ and experiments based on the binding of the hydrophobic probe 1-anilino-8-naphthalene sulphonic acid (ANS) and the susceptibility to proteinases¹⁶ that the protein substrate bound to GroEL resembles a molten globule state.

This result is substantiated by comparison of the widths of the peaks arising from the +12 charge state in the mass spectra of the free molten globule and chaperone-bound states with those of the native (holo) protein and a fully exchanged (unprotected) state 20 min after the initiation of exchange (Fig. 5*c*).

METHODS. 3SScam-BLA⁴⁷ was a generous gift from T. Creighton (EMBL, Heidelberg, Germany). For this experiment, lyophilized apo 3SScam-BLA was deuterated by dissolving it in D₂O, followed by 1 h incubation at room temperature. The solution was then adjusted to pH 5, and reconstituted to 140 μM by ultrafiltration. Deuteration and preparation of the other samples was as described in the legends to Figs 2–4. Exchange was initiated by 10-fold dilution of the protein samples into water pH 5.0, 4 °C. Mass spectra were recorded as described in the legend to Fig. 2. Each experiment was repeated at least three times, and reproducibility of the data was confirmed.

Similar to the previous observation for the acid-induced molten globule state of BLA, the distribution of states populated by the bound protein is also significantly narrower than that expected for a large number of species with different degrees of protection ranging from that of the fully protected (N) state to the unprotected (D) state. Indeed, conformations protected to the extent of the N and D states contribute only marginally (<10% of the total population) to the observed protection in the chaperone-bound protein. Even more dramatic, however, is the observation that the peak width of the chaperone-bound and free molten globule states are virtually identical (width at half height 30 and 33 mass units, respectively). This indicates that the two partially folded states are very similar, not only in the distribution of conformations sampled but also in the degree of protection of individual species within each population. The results demonstrate, therefore, that the molten globule state of 3SScam-BLA mimics closely the conformation of the chaperonin-bound form.

In all of the molten globule states of α -lactalbumin studied in detail by hydrogen exchange methods thus far^{25,27,41}, as well as in intermediates populated during the kinetic folding of hen lysozyme²⁹, the majority of amides protected from hydrogen exchange reside in the α -domain, which is persistently structured in these states in the absence of a stably structured β -domain. We suggest, therefore, that the α -domain is also persistently structured in the chaperonin-bound state of α -lactalbumin and that protection of this domain accounts virtually entirely for the degree of protection observed. By comparison with the well characterized molten globule states of α -lactalbumin with four disulphide bonds formed at acid pH^{25,27,41} and at pH 6 in the absence of calcium¹⁹, the molten globule states of 3SScam-BLA and GroEL-bound [3SS] BLA are more weakly protected from

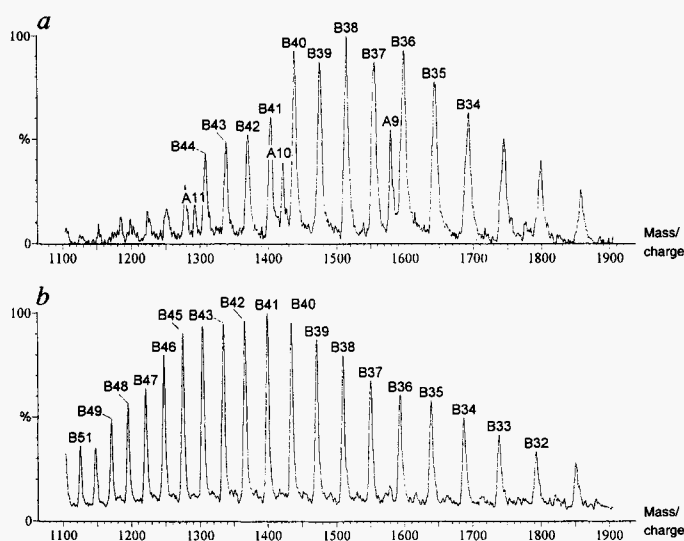


FIG. 6 ESI mass spectra monitoring hydrogen exchange of the GroEL:[3SS] BLA complex at pH 5, 20 °C, 23 min (a) and 55 min (b) after dilution into H₂O.

METHODS. The GroEL:[3SS] BLA complex was prepared as described in the legend to Fig. 4. Exchange was initiated by a 10-fold dilution into water/formic acid pH 5.0 at 20 °C. Mass spectra were recorded from water, without source heating, and were calibrated against hen egg-white lysozyme. The mass spectrum was obtained within the mass/charge range of $1,500 \pm 400$ and data represent the average of 10 scans.

exchange, consistent with significant destabilization of the molten globule state by reduction of one of the four disulphide bonds^{46,49}. Our results suggest, therefore, that molecular recognition by GroEL involves states which are substantially hydrophobically collapsed, with substantial secondary structure and few persistent tertiary interactions, but which are highly

dynamic. In this manner they resemble disordered molten globule states²⁰ akin to the collapsed state of hen lysozyme formed within the first few milliseconds of kinetic refolding experiments *in vitro*^{28,29}. This conclusion is in accord with the recent observation¹⁹ that the molten globule state of BLA with four native disulphide bonds does not bind to GroEL, presumably because it represents a more ordered molten globule state which resembles intermediates formed late during folding *in vitro*^{28,29}. We propose, therefore, that it is during the very early stages of folding that GroEL exerts its major influence during folding in the cellular environment.

Discussion

Our current results have demonstrated the feasibility of detecting hydrogen exchange protection in a range of conformational states, including partially folded labile structures, by ESI-MS. This approach has been applied directly to the study of a partially folded protein bound within the GroEL central cavity, and shown to yield new information about the conformational properties of the bound ligand. This technique is particularly powerful because it enables us to probe the structure of both the chaperone and its substrate without prior dissociation of the complex in solution. Dissociation of the complex to its monomeric components was crucial to quantify the number of protected deuterons in the bound ligand, but we show that this occurred only after introduction of the sample into the mass spectrometer, where hydrogen exchange does not take place. The power of the method described in this article lies not only in its ability to allow direct observation of large protein complexes which are well beyond the molecular mass range amenable to analysis by NMR, and of states presumably too dynamic to be visualized by X-ray crystallography, but also in the minimal quantities of sample (<1 nmol) required. ESI-MS, when coupled with hydrogen exchange labelling techniques, therefore, offers a new and exciting perspective, not only in the study of the mechanism of action of GroEL, but also of other noncovalent protein complexes, including the plethora of molecular chaperones that help protein folding *in vivo*. □

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Detection of a γ -ray burst of very long duration and very high energy

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ALTHOUGH γ -ray bursts (GRBs) have been known for more than 20 years, their origin remains mysterious¹. Suggestions have ranged from nearby colliding comets² to merging neutron stars at cosmological distances³. The lack of any counterpart at wavelengths other than X-rays and γ -rays has posed a major problem in identifying the source of GRBs⁴. Although in principle the distribution of energies among the burst photons, as well as their light curves, may be used to constrain the potential sources, this has proved difficult in practice⁵. Here we present the observation of a particularly energetic burst with a duration of 90 minutes, which includes the detection of an 18-GeV photon. For comparison, typical GRBs emit photons in the energy range between a few keV and a few tens of MeV, and last a few tens of seconds^{6,7}. The extended nature of this burst holds out the hope that there will be opportunities for telescopes operating at other wavelengths to detect a GRB source while it is still active, thus providing further constraints on the source's identity and properties.

This event occurred on 1994 February 17 at 82,962 s UT. Figure 1 shows the location of the burst as determined by the Compton Telescope (COMPTEL)⁸, the Energetic Gamma-Ray Experiment Telescope (EGRET) and Interplanetary Network (Ulysses/Burst And Transient Source Experiment, BATSE). Figure 2 shows the Ulysses 25–150 keV counting rates with 1-s resolution. The burst is clearly visible as a 180-s event at the start of the plot. Also shown are the energies of each of the EGRET spark-chamber photons from the source region as a function of time (see Table 1). The spark chamber recorded 10 photons while the low-energy burst was in progress. Following this, an additional 18 photons were recorded for ~5,400 s, including an 18-GeV photon ~4,500 s after the low-energy emission had ended. The EGRET background rate above 30 MeV consistent with this direction (95% confidence contour of Fig.

TABLE 1 EGRET photon energies and arrival times

Seconds of day 1994 Feb 17/18 UT	Energy $\pm$ error (MeV)	
82,985.7	231	59
83,004.7	2,665	315
83,024.0	75	30
83,027.7	174	20
83,039.5	181	49
83,049.3	3,382	421
83,056.1	45	16
83,074.5	72	13
83,075.5	262	65
83,076.0	44	17

83,175.4	105	15
83,344.0	98	30
83,388.0	43	23
83,392.5	129	39
83,476.7	118	16
83,483.0	137	17
83,753.5	60	30
83,808.0	89	13

01,318.3	18,390	3,860
01,335.9	56	50
01,421.7	93	53
01,482.4	65	13
01,647.6	69	12
01,688.7	49	10
01,753.7	94	34
01,909.9	99	14
02,142.1	75	12
02,146.9	36	12

Dashed line indicates end of low-energy burst. First and second dotted lines indicate interruption of data collection between 83,880 and 1,200 s UT, and 2,390 and 7,800 s UT, due to Earth-occultation and by the lack of coverage by the tracking and data relay satellite system.

1) is ~1 per 460 s, determined over the two-week period of this telescope pointing. Thus 0.39 photons were expected during the interval when the low-energy burst was in progress; the Poisson probability of observing 10 or more is $P = 1.5 \times 10^{-11}$. In the first interval of delayed emission (after the low-energy burst had ceased and before Earth occultation) 1.8 events are expected and 8 are observed ($P(\geq 8) = 6 \times 10^{-4}$); in the second interval, 2.9 are expected and 10 are observed ($P(\geq 10) = 9 \times 10^{-4}$). For the entire period of delayed emission, 4.7 are expected and 18 are observed ($P(\geq 18) = 2 \times 10^{-6}$). To verify that these increases could not be due to systematic effects, we have counted the number of photons observed in each of 149 orbits about this time and the associated probabilities of obtaining them; the distribution displays no anomalies. In addition, we verified that in the time immediately preceding the burst, the background was

TABLE 2 EGRET spark-chamber observations of γ -ray bursts

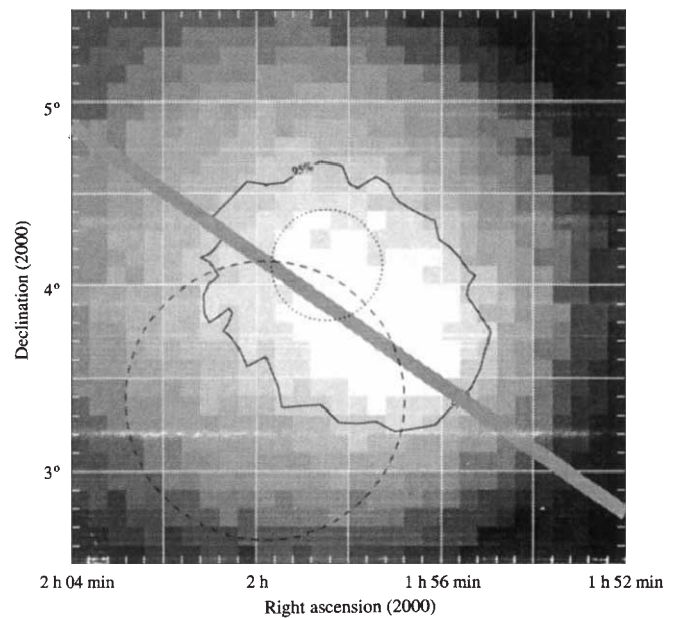
Date	Maximum energy (GeV)	Duration of high-energy emission	Event-averaged MeV spectrum*
1991 May 3 (refs 13, 20)	10	84 s	$E^{-2.2}$
1991 June 1 (refs 13, 21)	0.314	200 s	$E^{-3.7}$
1993 Jan 31 (refs 12, 13)	1.2	100 s	$E^{-2.0}$
1994 Feb 17†	18	1.5 h	$E^{-2.6}$
1994 Mar 1‡	0.16	30 s	$E^{-2.5}$

* E is energy; this column gives the best-fitting functional shape for the energy spectrum.

† This work.

‡ E.S. et al., manuscript in preparation; delayed emission not observed.

FIG. 1 Localizations of the 1994 Feb 17 burst. Solid line, EGRET 95% confidence contour, obtained by maximum-likelihood analysis of the first 10 photons; small dotted circle, error radius for the 18-GeV photon; large dashed circle, the 2σ COMPTEL burst location. The arc is the preliminary Interplanetary Network localization, resulting from the Ulysses and BATSE observations. It is centred at right ascension (2000) 328.736° , declination (2000) 51.947° , with radius $69.334^\circ \pm 0.05^\circ$. The BATSE error circle encompasses these localizations.



at its normal rate. The rate of detection of photons with energies ≥ 18 GeV cannot be determined reliably in this manner, as only one other such photon was detected in this two-week pointing. Thus the combined exposure of the first 27 months of the mission to galactic latitudes $|b| > 10^\circ$ (97 photons) was used to arrive at the probability of detecting a >18 -GeV photon in the 5,400-s interval: 5×10^{-6} . As indicated in Fig. 2, the source position

was Earth-occulted for $\sim 3,700$ s; however, as the EGRET count rates before and after occultation appear to be consistent with a constant rate, it is possible that continuous emission was present for the entire 5,400-s period after the low-energy burst.

Figure 3 shows the background-subtracted energy spectrum for the first 180 s. The fluences, or time-integrated fluxes, at various times and in various energy intervals give a measure of

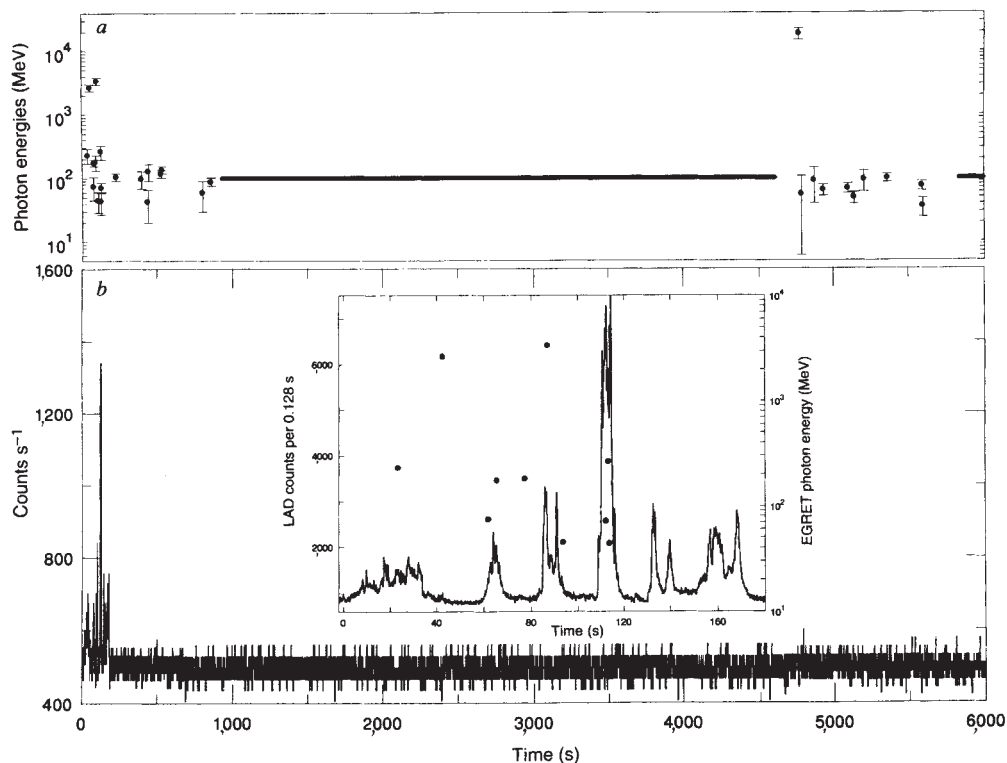
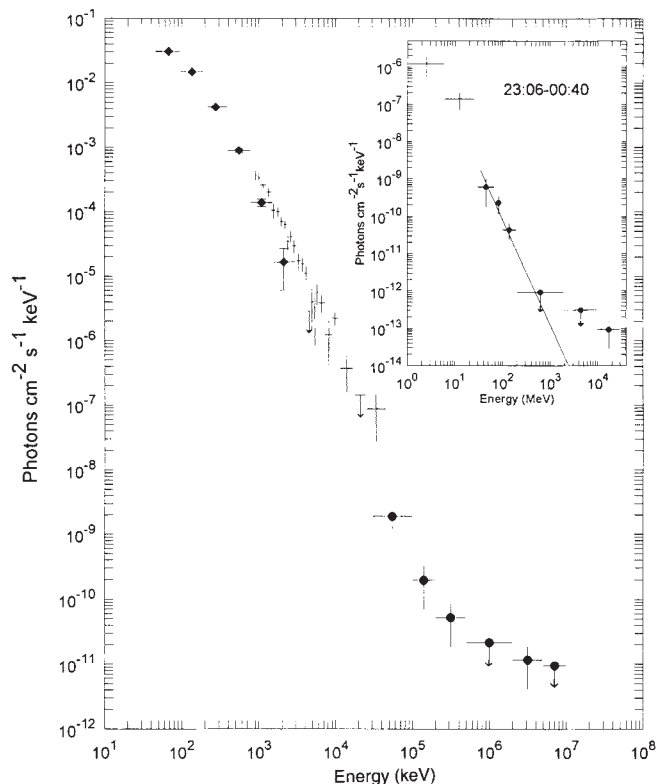


FIG. 2 *a*, EGRET spark energies as a function of time; horizontal lines indicate periods where no data is available. The EGRET spark chamber¹⁷ covers the 30 MeV to 30 GeV energy range, recording the time, energy and arrival direction of individual photons. *b*, The Ulysses 25–150 keV count rates as a function of time. The 180-s burst is evident at the start. The Ulysses γ -ray burst detector consists of two hemispherical scintillators with projected area 20 cm^2 in any direction,

covering the 25–150 keV energy range¹⁸. Because Ulysses operates in interplanetary space with $>95\%$ data recovery, it is not subject to interruptions such as Earth occultation or South Atlantic Anomaly passages. Inset, expanded plot of the first 200 s as observed by BATSE, showing the arrival times and energies of the EGRET photons. The BATSE large area detectors (LADs) are $2,025\text{-cm}^2$ scintillators covering the 30–1,900 keV energy range¹⁹.

FIG. 3 Main figure, deconvolved BATSE spectroscopy detector (SD) and EGRET energy spectra of the first 180 s of the burst. The BATSE SDs are 5" diameter by 3" thick scintillators which measure the energy spectra of bursts in 256 channels typically between 30 and 2,000 keV (ref. 19). The energy ranges and best fits are: SDs (crosses with diamonds) $41\text{--}2,961\text{ keV}$, $dN/dE = (1.95 \pm 0.02) \times 10^{-2} (E/100\text{ keV})^{-1.215 \pm 0.02} \exp(-E/(838 \pm 63)\text{ keV})$ photons $\text{cm}^{-2}\text{ s}^{-1}\text{ keV}^{-1}$, where N is the number of photons and E is the energy in keV; EGRET total absorption shower counter (TASC), a NaI scintillator with effective area $\sim 1,500\text{ cm}^2$, which accumulates 256 channel 1–200 MeV energy spectra every 32.768 s (ref. 20) (plain crosses), $0.94\text{--}92\text{ MeV}$, $dN/dE = (3.6 \pm 0.26) \times 10^{-4} E^{-2.5 \pm 0.08}$ photons $\text{cm}^{-2}\text{ s}^{-1}\text{ keV}^{-1}$; spark chamber (crosses with circles), $30\text{--}4,000\text{ MeV}$, $(2.91 \pm 0.97) \times 10^{-7} (E/128\text{ MeV})^{-2.08 \pm 0.56}$ photons $\text{cm}^{-2}\text{ s}^{-1}\text{ MeV}^{-1}$. The EGRET 30 MeV to 5 GeV fluence, obtained by integrating the spectral fit, was $2 \times 10^{-5}\text{ erg cm}^{-2}$; as the fit is based on only 10 photons, this number was also calculated by a Monte Carlo method, which gave $(1.5 \pm 1.3) \times 10^{-5}\text{ erg cm}^{-2}$. Inset, deconvolved TASC and spark-chamber spectra of the delayed emission, with different binning. The best fit to the 30–30,000 MeV spark-chamber data is $(1.3 \pm 0.4) \times 10^{-8} (E/86\text{ MeV})^{-(2.83 \pm 0.64)}$ photons $\text{cm}^{-2}\text{ s}^{-1}\text{ MeV}^{-1}$. The highest-energy point is based on the single 18-GeV photon and is consistent with the power-law fit at the 99% confidence level. The fluence in this spectrum at $>30\text{ MeV}$, obtained by integrating the power-law fit, is $7 \times 10^{-6}\text{ erg cm}^{-2}$. The uncertainties in the fitting parameters influence this number considerably; for example the fluence of the 18-GeV photon alone is $\sim 7 \times 10^{-5}\text{ erg cm}^{-2}$.



the overall energetics of this event. The total fluence above 20 keV is $(6.6 \pm 2.7) \times 10^{-4}\text{ erg cm}^{-2}$, obtained by fitting the spectrum observed by the BATSE large area detectors (G. Pendleton, personal communication). This is the third-largest fluence of ~ 800 BATSE bursts to date. Figure 3 inset shows the background-subtracted 30 MeV to 30 GeV EGRET spectrum for the 5,400-s period following the burst. If the $>30\text{-MeV}$ emission continued during the Earth-occulted period at a constant level, the fluence in this component would have been $1.5 \times 10^{-5}\text{ erg cm}^{-2}$, or 2% of the total. The continuous Ulysses and BATSE data have been searched for evidence of accompanying low-energy emission of various durations during the 5,400 s following the burst. None was found. From the BATSE occultation data⁹ a limit to the 20–300 keV emission in any 5,400-s unocculted interval during the extended emission is $2.7 \times 10^{-5}\text{ erg cm}^{-2}$. Thus whereas the $>30\text{ MeV}$ emission represents only 3% of the energy during the first 180 s, it accounts for over 50% of the energy of the extended emission.

To put these observations into perspective, photon spectra in the $\sim 30\text{--}1,000\text{ keV}$ range can be fitted to a function which turns over at energies typically $<200\text{ keV}$ (ref. 10). There is a tendency for the energy spectra to evolve in conjunction with the light curve, with harder spectra at the peaks, and this is indeed the case for the first 180 s of this event. The maximum duration is

$\sim 1,000\text{ s}$ (ref. 11) at these energies. Before this event, the highest energy observed in a burst in conjunction with low-energy emission was 1.2 GeV (ref. 12, Table 2); a tendency for the high-energy emission to lag that at lower energies had been noted, with the highest overall energy being 10 GeV (ref. 13).

Mészáros and Rees¹⁴ and Katz¹⁵ have suggested that this delayed high-energy emission may be the consequence of the collision between an expanding shell of debris, created by a binary neutron-star merger, and an external cloud of matter. Such a delay, however, is not necessarily a signature of cosmological origin. EGRET has observed 1-GeV emission from the Sun with a delay of 1–4 h (ref. 16) following a flare.

Delayed, very-high-energy emission from γ -ray burst sources may now be considered a well established fact, but it is not yet clear whether it accompanies all bursts. EGRET has had some exposure to over 150 BATSE bursts. The five events that it has unambiguously detected have the highest BATSE peak fluxes and/or fluences. However, the summed high-energy emission from the numerous weaker events may yet prove to be detectable. The emission delay provides a 'window of opportunity' which may be used by TeV detectors (such as air Cherenkov telescopes or the Milagro air-shower array) to change viewing directions or operating modes. The detection of unattenuated emission at these energies would serve as a crucial test for cosmological models of burst sources. $\square$

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Record of fluid-rock interactions on Mars from the meteorite ALH84001

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ALLAN HILLS (ALH) 84001 is the most recently recognized¹ member of a suite of meteorites—the SNCs—that almost certainly originated on Mars². Several factors distinguish ALH84001 from the other SNC meteorites. Preliminary studies^{3,4} suggest that it may be older than other martian meteorites. Moreover, it contains abundant, zoned domains of calcium-iron-magnesium carbonate that are indigenous to the sample¹ and thus may hold important clues regarding near-surface processes on Mars and the evolution of the martian atmosphere. We report here analyses of the carbon and oxygen stable-isotope compositions of the carbonates that place constraints on their formation conditions. Our results imply the presence of at least two chemically distinct carbonates—one Ca,Fe-rich, the other Mg-rich—that are enriched in ¹³C relative to terrestrial carbonates ($\delta^{13}\text{C} \approx +41\text{‰}$), consistent with martian atmospheric CO₂ as the carbon source. The oxygen isotope compositions of the carbonates indicate that they precipitated from a low-temperature fluid in the martian crust. Combined with textural and bulk geochemical considerations, the isotope data suggest that carbonate deposition took place in an open-system environment in which the ambient temperature fluctuated.

The mineralogy of ALH84001 is dominated by orthopyroxene, but ~1 vol% consists of orange-coloured carbonates (Fig. 1). Mittlefehldt¹ suggested that the carbonates were formed on Mars, and, on the basis of cation composition, deduced a formation temperature of up to 700 °C, requiring development at depth. Using carbon and oxygen stable-isotope measurements,

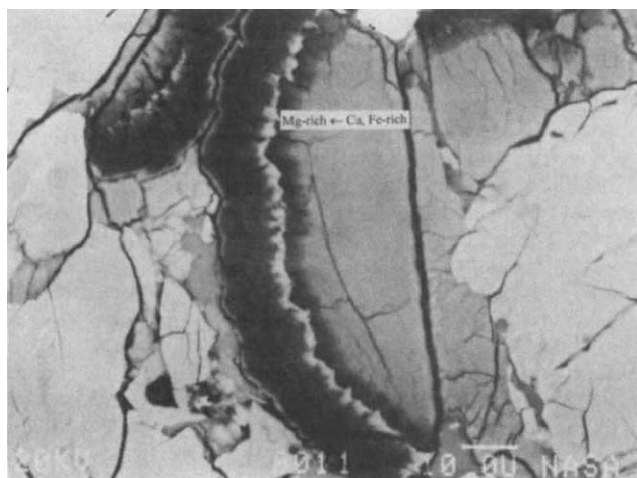


FIG. 1 Back-scattered electron image of a complex zoned carbonate in ALH84001. The Mg-rich carbonate (dark) has crystal faces that project outwards, suggesting growth from the centre of the grain to the outer margin. These are then overgrown in turn by a further band of Ca,Fe-rich carbonate (white). Scale bar, 10 μm

we examine an alternative formation mechanism: that the carbonates are metastable, low-temperature crustal deposits precipitated during open- or closed-system aqueous alteration (any terrestrial weathering components occur in very minor quantities⁵). If the minerals are non-terrestrial alteration products, formed from fluids in equilibrium with the martian atmosphere, they should prove instrumental in describing both the operation of near-surface processes on Mars and the evolution of the martian atmosphere.

Most carbonate grains within ALH84001 are compositionally zoned, from Ca- and Fe-rich to Mg-rich (Fig. 1). Zoning is oscillatory, indicating that precipitation of the carbonates was not from a single event, but that either multiple influxes of fluid occurred, or ambient temperature fluctuated. Textural evidence suggests that the more Ca,Fe-rich carbonates crystallized before the Mg-rich zones. The isotopic compositions of a hand-picked carbonate separate, determined by acid digestion, are given in Table 1a. The acid treatment was carried out in stages, in order to attack the different carbonates: Ca,Fe-carbonates react faster than Mg carbonates^{6,7}. Carbon dioxide produced in the first four hours of reaction is less enriched in ¹³C and ¹⁸O (far less so in the case of the latter) compared with two further extractions at 16 and 24 h, suggesting that the Ca,Fe- and Mg-carbonates have different isotopic compositions. Taking the weighted means of isotope data from the last two steps as representative of Mg-rich carbonate, the isotopic compositions of the Ca,Fe-bearing phase can be calculated (Table 1b).

The oxygen isotope compositions of the carbonates ($\delta^{18}\text{O} \approx +13.3$ to $+22.3\text{‰}$) are substantially different from the silicates of the host rock ($+4.6\text{‰}$; ref. 8). If the carbonates had formed at 700 °C, as inferred from cation distributions¹, oxygen isotopic equilibrium should have been attained⁹, resulting in $\delta^{18}\text{O}$ values for the carbonates of $+6$ to $+8\text{‰}$. This discrepancy between isotopic and chemical data can be explained if the carbonates were deposited from a fluid at low temperatures. Alternatively, primary carbonates exchanged isotopically with the fluid to lower temperatures than the orthopyroxene. In either case, interaction with hydrothermal fluids is required, which may have resulted in chemically disequilibrated carbonates.

TABLE 1 Isotopic composition of ALH84001 carbonate

(a) Measured values*			
Aliquot	$\delta^{13}\text{C}$ (‰)	$\delta^{18}\text{O}$ (‰)	Yield (μg of C)
0–4 h	+40.3	+16.4	4.32
4–16 h	+41.9	+22.6	2.55
16–24 h	+41.5	+21.4	0.79
(b) Calculated endmembers†			
Composition	$\delta^{13}\text{C}$ (‰)	$\delta^{18}\text{O}$ (‰)	Yield (μg of C)
Ca,Fe-rich	+39.5	+13.3	1.50
Mg-rich	+41.8	+22.3	6.16

* Individual carbonate grains were hand-picked from 80 mg of gently crushed ALH84001, making no attempt to distinguish between different generations. Carbon and oxygen isotopic compositions of the material were determined by digestion in orthophosphoric acid at 75 °C (refs 24, 25). CO₂ was continuously removed from the reaction vessel during the first 4 h of the reaction. Sequential aliquots were then extracted from the closed vessel after 16 and 24 h. Slight shifts in isotopic composition between 'continuous extraction' and 'sealed vessel' mode were characterized and appropriate corrections applied²⁵. Results are reported in the δ notation, relative to PDB for carbon and SMOW for oxygen. CO₂ yields were determined by capacitance manometer.

† The isotopic composition of the Mg-rich endmember was taken as the weighted mean of the 4–16 h and 16–24 h aliquots (see a), from which the Ca,Fe-rich endmember was calculated, assuming first-order dissolution kinetics for the acid digestion process.

The $\delta^{18}\text{O}$ of carbonate is dependent on precipitation temperature and molar $\text{H}_2\text{O}/\text{CO}_2$ ratio of the fluid (Fig. 2)^{10,11}. Several assumptions are made in constructing this figure: (1) the initial composition of martian volatiles has not been modified by subsequent processing; (2) the minimum temperature experienced during carbonate precipitation is 0 °C (that is, the fluid is $\text{H}-\text{O}-\text{C}$ only, and not a brine), and (3) the water/rock ratio is very much less than 1 (a reasonable supposition, given that hydrated mineral phases have yet to be observed in the parent rock). Although these assumptions may be subject to review, two endmember models—(1) open system (variable temperature, constant $\text{H}_2\text{O}/\text{CO}_2$) and (2) closed system (constant temperature, variable $\text{H}_2\text{O}/\text{CO}_2$)—can still be considered, and Fig. 2 used to deduce approximate formation temperatures for the Ca, Fe- and Mg-rich phases.

In an open system, $\delta^{18}\text{O}$ of the carbonates at the minimum assumed temperature of 0 °C suggests a $\text{H}_2\text{O}/\text{CO}_2$ ratio of ~ 0.58 (Fig. 2). If the Mg-rich carbonate mantles were deposited under these conditions, then Ca, Fe-carbonates (core material) must have been precipitated at ~ 80 °C to account for their lower $\delta^{18}\text{O}$ value. If $\text{H}_2\text{O}/\text{CO}_2$ is not fixed, as might be the case in a completely open system, with fluid constantly percolating through the rock, then a smaller temperature difference can account for the calculated variation in $\delta^{18}\text{O}$ of the carbonates.

The observed oxygen isotopic results are also compatible with carbonate precipitation in a closed system influenced by Rayleigh fractionation: as carbonate is precipitated, the residual fluid pool becomes enriched in ^{18}O , resulting in higher $\delta^{18}\text{O}$ values for subsequent deposits. However, carbon data are not wholly consistent with this model. There is a crossover in carbon isotope fractionation between CO_2 and carbonate at temperatures around 200 °C (ref. 12). Below 200 °C, as carbonate precipitates, the residual fluid pool becomes progressively depleted in ^{13}C , implying that $\delta^{13}\text{C}$ of late-stage carbonates will be lower than those precipitated earlier. As this is not the case (Table 1b), precipitation above 200 °C is predicted, temperatures perhaps rather high for near-surface aqueous processes on Mars. Finally,

as the changes in cation composition of the carbonates also cannot be derived within a closed system, it seems inappropriate to employ this model to describe precipitation of the carbonates in ALH84001.

The $\delta^{13}\text{C}$ values of carbonates from ALH84001 indicate a probable source of the CO_2 for the minerals. A $\delta^{13}\text{C}$ of $\sim +41\text{‰}$ is outside the range of terrestrial carbonates^{13,14}. It is also one of the highest confirmed in an SNC meteorite; indeed, $\delta^{13}\text{C}$ of $+41.3\text{‰}$ was also measured for ALH84001 by the alternative technique of stepped combustion¹⁵. A higher value ($\delta^{13}\text{C} \approx +49\text{‰}$) was reported during extended dissolution of another SNC meteorite, Nakhla, but the amounts of gas were very small, and isotopic data considered uncertain¹⁶; they now may be considered a true measurement. The relative similarity in $\delta^{13}\text{C}$ for the Ca, Fe- and Mg-carbonates reflects the probability of a single source for the carbon, most likely to be martian atmospheric CO_2 , for which $\delta^{13}\text{C}$ has been calculated at $+36 \pm 10\text{‰}$ (ref. 16), and measured at $> +27\text{‰}$ (ref. 17).

Based on the interpretations above, Mars may have been a warm and wet planet, where volatile species (CO_2 and H_2O) could freely exchange. After early outgassing of the planet, low-temperature isotopic re-equilibration and non-thermal gaseous escape produced an atmosphere composed of CO_2 enriched in ^{13}C , and H_2O depleted in ^{18}O (ref. 18). As a consequence of these interactions, carbonates which formed near the surface presumably contain isotopic records of secular change related to their age. In the foregoing discussion, estimates for the $\delta^{13}\text{C}$ of martian atmospheric CO_2 were based on analyses of gas trapped in EETA79001, rather than on the values obtained by the Viking spacecraft, which are only poorly-constrained ($0 \pm 100\text{‰}$)¹⁹. EETA79001 has a formation age of ~ 180 Myr (ref. 20), hence trapping of the gas occurred relatively recently. As the carbonates in ALH84001 display $\delta^{13}\text{C}$ values similar to those of the assumed atmosphere, then either hydrothermal alteration of ALH84001 occurred at a similar time to that at which EETA79001 trapped atmospheric CO_2 (that is, the carbonates are also relatively recent) or $\delta^{13}\text{C}$ of atmospheric CO_2 has not changed between precipitation of the carbonates and trapping of gas in EETA79001. If the carbonates prove to be ancient³, it implies that the martian atmosphere has undergone only minimal isotopic fractionation over an extended time interval. This conclusion is also supported by hydrogen isotope data²¹, and has significant implications for the interpretation of martian atmospheric evolution.

There is little textural evidence in ALH84001 for the infiltration of fluids along cracks or other migration pathways. Most of the carbonate grains pre-date the shock event that created crushed zones within the meteorite¹, hence these zones cannot be proposed as channels for fluid percolation. In order to introduce carbon and oxygen into the crust for precipitation as carbonate, without significant isotopic exchange with host rock, surface-equilibrated fluids must have been transported at low temperature to the site of carbonate deposition. Hydrothermal cells are the most likely candidates for fluid transport, although the driving mechanism behind such circulation is poorly understood²².

Accurately constraining formation conditions for the carbonates in ALH84001 is not yet possible using isotope data from the carbonates alone, but requires isotopic compositions for other components within the system, for example, $\delta^{18}\text{O}$ of water-bearing minerals. Even so, employing an open-system model yields low formation temperatures (0 to $+80$ °C) and a range of fluid compositions for precipitation of martian carbonates isolated from the ALH84001 meteorite. The mean $\delta^{13}\text{C}$ value of $\sim +41.3\text{‰}$ is compatible with the source of carbon being CO_2 in the martian atmosphere, providing evidence of a circulation system which links the martian atmosphere, hydrosphere and lithosphere. The processes recorded in ALH84001 may help to answer the question of how warm and how wet was early Mars²³. □

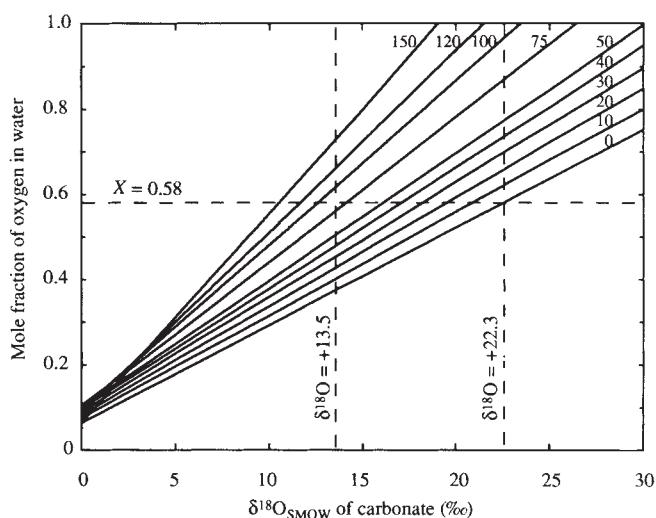


FIG. 2 Variation of martian $\text{H}_2\text{O}/\text{CO}_2$ ratio (expressed as the mole fraction, X , of oxygen in water) with $\delta^{18}\text{O}$ of carbonates for a range of temperatures (shown on the lines, °C) appropriate for the martian surface. The figure was constructed from a combination of expressions giving the fractionation in oxygen between CO_2 (gas)–water and carbonate–water, following the models of refs 10 and 11. Implicit in the model is the assumption that the starting reservoir of volatiles was produced by high-temperature degassing of Mars, and that the volatiles have not subsequently been modified. ($\delta^{18}\text{O}$ expressed relative to the SMOW standard).

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Surface spectroscopy with high spatial resolution using metastable atoms

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THE study of surface phenomena is in large part dependent on spectroscopic techniques that are sensitive to only the outermost few layers of the material under consideration. A variety of such techniques are now available, some of which also have the benefit of high spatial resolution^{1–3}. Here we show that metastable atoms—that is, atoms in long-lived excited states—can be used as a sensitive surface probe with high spatial resolution. In contrast to electrons or photons, metastable atoms cannot penetrate into a solid; instead, they are de-excited readily following interaction with the surface electronic orbitals^{4,5}. The de-excitation process is accompanied by the emission of electrons, the energy spectrum of which provides fundamental information about the electronic properties of the surface. High spatial resolution (in the present case, about 5 μm) is achieved by detecting electrons that have been emitted from only a small area of the sample. As the metastable atoms have only thermal kinetic energies, they are essentially non-destructive, making them ideally suited to probing fragile surfaces such as organic layers and biological specimens.

In contrast to the charged-particle or photon beam, the metastable-atom beam can not be converged and therefore the electron emission image produced by metastables is difficult to obtain. To date, only an emission microscope image by fast argon atoms mixed with metastable ones has been reported⁶. When a metastable atom such as $\text{He}^*(1s2s, 2^1S)$ strikes an ordinary metal or semiconductor surface, the $2s$ electron of He^* tunnels into an empty level of the surface (resonance ionization) and the He^+ ion thus formed is then neutralized by an electron from the surface with simultaneous emission of another surface electron (Auger neutralization)⁷. On the other hand, on an insulator surface the resonance ionization process is suppressed because the $2s$ level of He^* is within the energy gap of the insulator. In this case, an electron from the filled orbital of the surface transfers to the $1s$ hole of He^* with the simultaneous ejection of the $2s$ electron (Penning ionization or Auger de-excitation)⁷. Thus analysing the energy of electrons ejected through the resonance ionization + Auger neutralization process, and through the

Penning ionization process, gives information on the distributions of vacant orbitals and occupied orbitals exposed outside the surface layer, respectively^{8,9}. In addition to this extreme surface sensitivity, metastable atoms with electron spin such as $\text{He}^*(2^3S)$ have the ability to probe the long-range spin ordering on the solid surface¹⁰. Hence the electron emission microscope using metastable atoms, although difficult to construct, will become a useful technique in surface science.

The instrument used in our experiment consists of a specimen preparation chamber, a main chamber which is equipped with systems for LEED (low-energy electron diffraction) and AES (Auger electron spectroscopy), and an electron microscope (Fig. 1). The pressure in the instrument is $\sim 10^{-8}$ Pa. The microscope is available for low-energy electron microscopy (LEEM)¹¹, photoelectron emission microscopy (PEEM), and metastable electron emission microscopy (MEEM), depending on the incident beam. The details of the instrumentation will be published elsewhere.

For MEEM, the specimen is irradiated with an $\text{He}^*(2^3S)$ atom beam. Metastable helium atoms (2^1S , 20.62 eV; 2^3S , 19.82 eV) are produced with a discharge tube basically similar to those described by Leasure, Mueller and Ridley¹², and Fahey, Parks

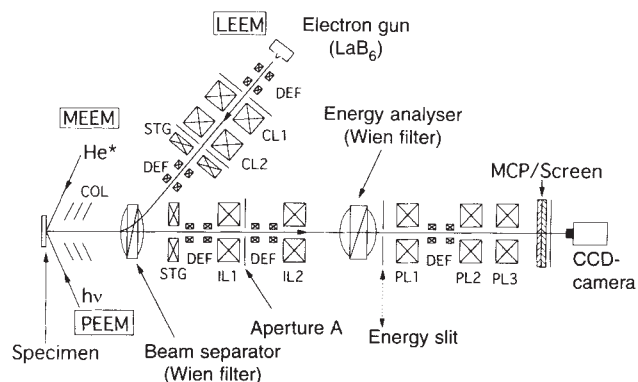


FIG. 1 Schematic diagram of the microscope for low-energy electron microscopy (LEEM), photoelectron emission microscopy (PEEM) and metastable electron emission microscopy (MEEM). For MEEM, electrons emitted from the specimen are accelerated to 10 keV and imaged by the electron optical system consisting of a cathode objective lens (COL), two intermediate lenses (IL1, IL2) and three projector lenses (PL1, PL2, PL3). The final image is observed with the fluorescent screen with two multi-channel plates (MCP) and stored in a video tape using the CCD (charge-coupled device) camera. Defectors (DEF) and stigmators (STG) are used to align the beam and to compensate the astigmatism. For the measurement of electron spectra, the energy slit is inserted and the energy of electrons emitted from a local area of the specimen is analysed by a Wien filter. The local area is selected by adjusting the aperture A. The energy resolution of the Wien filter estimated by electron optical simulation is 0.1 eV.

and Schearer¹³. But we obtained a very intense He* beam of 2×10^{16} atoms $\text{s}^{-1} \text{sr}^{-1}$ by using a Ta hollow cathode, instead of their needle cathodes, and evacuating the source chamber with a high-pumping-speed diffusion pump ($3,000 \text{ l s}^{-1}$). To obtain pure He*(2^3S) atoms, He*(2^1S) metastable atoms are quenched by a water-cooled He discharge lamp. A Grafoil specimen (consisting of graphite crystallites with their basal (cleavage) planes parallel to the foil plane) was cleaned by heating at 400°C for 16 h under ultra-high vacuum. Gold and ClAl-phthalocyanine (ClAlPc) films were made in the preparation chamber by vapour deposition onto Grafoil; the film thickness was controlled by a quartz oscillator monitor.

Figure 2a shows a metastable electron-emission micrograph of Au islands on a Grafoil film. The thickness of Au corresponds to one atomic layer. In the figure, the dark regions are due to Au islands, which indicates that the Au monolayer has a lower total emission yield than the graphite substrate when metastable atoms strike the surface. The image of Au islands is not clear because of the aberrations of the optical system (especially the cathode objective lens) and also of the diffusion of some gold atoms from edges of islands. The aberrations are due to the fact that all the energies of emitted electrons (~ 0 – 15 eV) are used to produce the image. We are now trying to find the optical condition for obtaining the energy-filtered image.

Figure 3a shows the metastable electron emission spectrum of the graphite substrate (Grafoil). The diameter of the probe area ϕ is $\sim 5 \mu\text{m}$. Electron emission from graphite by impact of metastable atoms is due to Penning ionization^{14,15}. Because one electron of the surface is responsible for this ionization process, each band in the electron emission spectrum can be correlated with a valence or conduction band, as in the case of the photoelectron spectrum. In Fig. 3a, two features at ~ 12 and 7.5 eV are due to the π valence bands and the peak at $\sim 3 \text{ eV}$ is ascribed to σ^* conduction bands^{14,15}. The effective resolution of the energy analyser is estimated to be 0.6 eV from the full-width at half-maximum of the σ^* bands.

Figure 3b shows the metastable electron emission spectrum of an Au island shown in Fig. 2a. The diameter of the probe area ϕ is also $\sim 5 \mu\text{m}$. In contrast to the case of graphite, the spectrum of Au has a broad structureless feature, because metastable atoms are de-excited through the resonance ionization + Auger

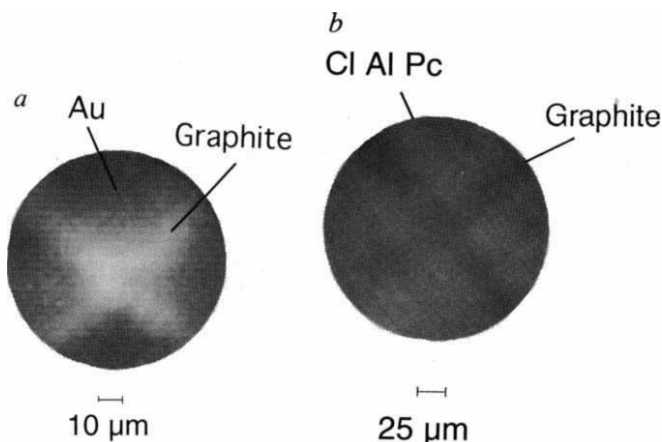


FIG. 2 a, Metastable electron-emission micrograph of Au islands (monatomic layers) on a Grafoil film. The light and dark areas are due to the graphite substrate and Au islands, respectively. The islands were grown by vacuum deposition at room temperature using a net mask of $100 \mu\text{m}$ width placed just above the Grafoil surface. b, Metastable electron-emission micrograph of ClAl-phthalocyanine (ClAlPc) monolayer islands on a Grafoil film. The light and dark areas are due to ClAlPc islands and the graphite substrate, respectively. The islands were grown by the same method as in the Au islands.

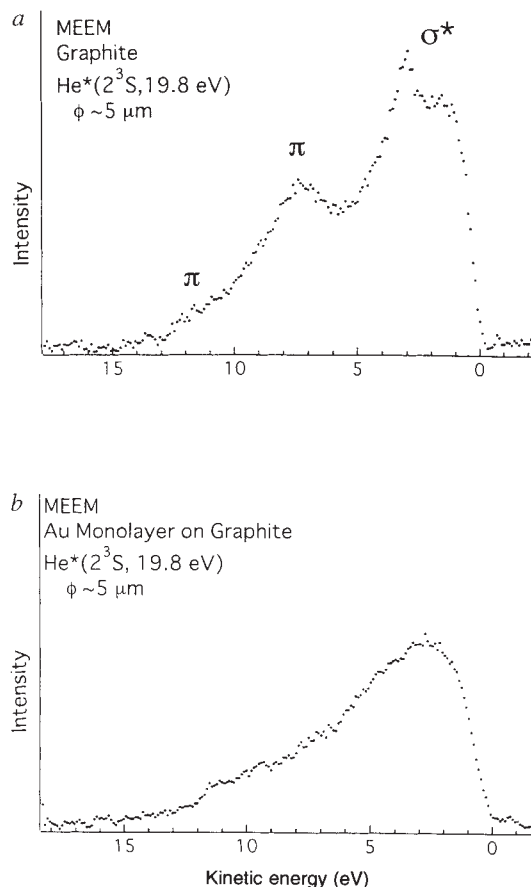


FIG. 3 Metastable electron emission spectra. a, The graphite substrate (Grafoil); b, an Au island (ϕ , diameter of the probe area).

neutralization process. In this case, the spectrum gives a broad self-convoluted feature, because the energy of the He⁺ ion formed in the resonance ionization process is shared by two electrons from the surface in the Auger neutralization process⁷. It is of note that the de-excitation process of the He* metastable atom switches from Penning ionization to resonance ionization + Auger neutralization, and the features of the graphite substrate disappear, when the surface of graphite is covered by one atomic layer of Au. This means that the metastable electron emission spectrum is selectively sensitive to the outermost surface layer.

Figure 2b shows a metastable electron-emission micrograph of monolayer islands of ClAlPc on Grafoil prepared at room temperature. In the figure the light regions are due to ClAlPc islands, showing that the ClAlPc monolayer has a higher total emission yield than the graphite substrate. Thus the image of one molecular layer is observed by MEEM. As in Au the vague image of ClAlPc islands is due to the aberrations and molecular diffusion. In fact the latter effect is so marked that the image of islands is hardly perceptible one day after their vacuum deposition.

Figure 4 shows metastable electron emission spectra of a ClAlPc monolayer (black dots) and a ClAlPc monolayer island (circles) on Grafoil (probe area $\phi \approx 5 \mu\text{m}$). Both spectra were measured three hours after vacuum deposition of the sample. The electron emission of both spectra are due to the Penning ionization process. In the spectrum of the monolayer, the bands around 13.3 , 11.5 , 9.9 and 8.7 eV are mainly related to the π (ring), π (benzene), $\text{Cl}(n_{\parallel})$ and $\text{Cl}(n_{\perp})$ orbitals. From the relative band intensity of the monolayer spectrum, phthalocyanine rings are considered to be oriented flat to the substrate with the

chlorine atom protruding outside the film surface (as shown at the top of Fig. 4). The same conclusion could be obtained in a wide spatial region from the study of ordinary Penning ionization electron spectroscopy, which also showed¹⁶ the change in the molecular orientation during layer-by-layer deposition of CIAIPc on graphite. In Fig. 4 the spectrum of the CIAIPc island (circles) is a mixture of the monolayer spectrum (black dots) and the Grafoil spectrum (Fig. 3b), indicating the diffusion of molecules of the CIAIPc island on Grafoil. Thus the spectrum obtained by MEEM probes the molecular diffusion in the monolayer, as well as the orientation of molecules at the outermost surface layer with high spatial resolution.

Emission spectromicroscopy, using metastable atoms as probes, provides information on the distribution of individual orbitals exposed outside the outermost layer of the surface. There are other electron spectroscopies (such as ultraviolet photoelectron spectroscopy, X-ray photoelectron spectroscopy and electron energy-loss spectroscopy) that allow detailed analyses of the structure and electronic state of the surface. These other methods, however, give information on the outermost surface layer mixed with that on inner layers, because photons and electrons used as probes penetrate below the surface. Metastable-atom electron spectroscopy is free from such an effect and provides direct information on the outermost layer, although the quantitative analysis of the data is rather difficult because of its complex ionization mechanism. Furthermore, metastable atoms

with thermal kinetic energy are essentially non-destructive (in fact, LEEM gave no sign of CIAIPc for monolayers on graphite owing to electron sputtering). The present technique, therefore, is suitable for the observation of more chemically fragile surfaces such as organic layers and biological specimens. □

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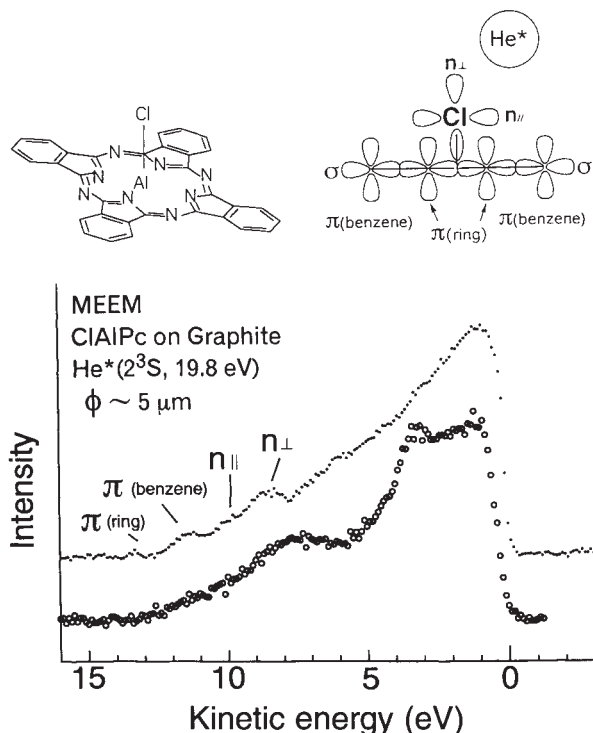


FIG. 4 Bottom, metastable electron emission spectra of a CIAIPc monolayer (black dots) and a CIAIPc monolayer island (circles) on Grafoil. Both spectra were measured 3 h after vacuum deposition of the sample. Top, schematic diagram showing the interaction between a He* metastable atom and molecular orbitals of CIAIPc. The $\pi(\text{ring})$ orbitals are distributed along the CN skeleton of the inner porphine-like ring, whereas $\pi(\text{benzene})$ orbitals are predominantly distributed in the benzene rings^{17,18}. The $\text{Cl}(n_{\parallel})$ and $\text{Cl}(n_{\perp})$ orbitals are the chlorine lone-pair orbitals distributed parallel and perpendicular to the phthalocyanine ring, respectively. In the monolayer, molecules are oriented flat to the substrate with the chlorine atom protruding outside, because He* atoms effectively interact with the $\text{Cl}(n_{\parallel})$, $\text{Cl}(n_{\perp})$ and $\pi(\text{benzene})$ orbitals giving stronger bands in the spectrum, while the $\pi(\text{ring})$ and σ orbitals (shielded by the Cl and the $\pi(\text{benzene})$ orbitals) show weaker bands.

Homogeneous catalysts based on silane dendrimers functionalized with arylnickel(II) complexes

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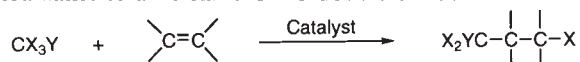
At the interface between heterogeneous and homogeneous catalysis there is great scope for the development of new materials that combine the advantages and/or minimize disadvantages associated with each of these classes. In particular there is a need for homogeneous catalysts with properties that allow their ready removal from a product-containing solution. One approach to such materials is to anchor homogeneous catalysts to soluble polymer supports¹; we have recently prepared such catalytic materials in which the active centre is an organometallic species^{2,3}. One disadvantage encountered when anchoring catalytic metal sites to polymers is the difficulty of accurate control of the number and location of these sites. Here we report an alternative approach—the synthesis of polysilane dendrimers (highly branched macromolecules^{4–6}) which are functionalized at their periphery with metal-containing catalytically active sites. These dendrimers show regiospecific catalytic activity for the Kharasch addition of polyhalogenoalkanes to carbon–carbon double bonds. It should be possible to remove the nanoscale catalytic macromolecules of this type from the solution of products using filtration methods.

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Recent advances with the incorporation of inorganic (metal) functionalities in dendrimers have included the synthesis of dendrimeric complexes with organoplatinum(IV) units⁷ and ruthenium(II) coordination centres⁸, as well as of some highly branched macromolecules with iron⁹ and ruthenium organometallic centres^{10,11}. In a previous report⁵, it was intimated that a dendrimer architecture might offer a means of better controlling the disposition of pendant metal-containing catalytic sites in soluble, polymer-based catalysts.

Our target molecules in this investigation are two organometallic polysilane dendrimers functionalized with four (**1**, generation 0; G0- $\{\text{SiMe}_2\text{-ArNiBr}\}_4$; $M_r = 2,269$; Fig. 1a) and twelve (**2**, generation 1; G1- $\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$; $M_r = 7,032$; Fig. 1b) diamino arylnickel(II) complexes on their periphery. The peripheral organometallic site, like that in diamino arylnickel(II) complexes **3** (Fig. 2), is one in which the metal centre is fixed by a metal-carbon σ -bond to the ligand system. This site has been shown, both in mononuclear complexes such as **3** (ref. 12) and when anchored in soluble siloxane polymers³, to be particularly effective for homogeneous catalysis of the Kharasch addition reaction of polyhalogenoalkanes to an olefinic C=C double bond:



Here X denotes a halogen, and Y denotes either H, halogen, CF_3 or other electronegative group. The alkenes used were, for example, methyl methacrylate, 1-octene and styrene. An attractive property of catalysts such as **3** is that they selectively produce the 1:1 adduct: that is, oligomeric or polymeric products are essentially absent.

The divergent synthetic route to the catalytic dendrimers **1** and **2**, outlined in Fig. 3, begins with the silane $\text{Si}(\text{CH}_2\text{CH}=\text{CH}_2)_4$ (generation 0; G0) and the silane dendrimer $\text{Si}\{\text{CH}_2\text{CH}_2\text{CH}_2\text{-Si}(\text{CH}_2\text{CH}=\text{CH}_2)_3\}_4$ (generation 1;

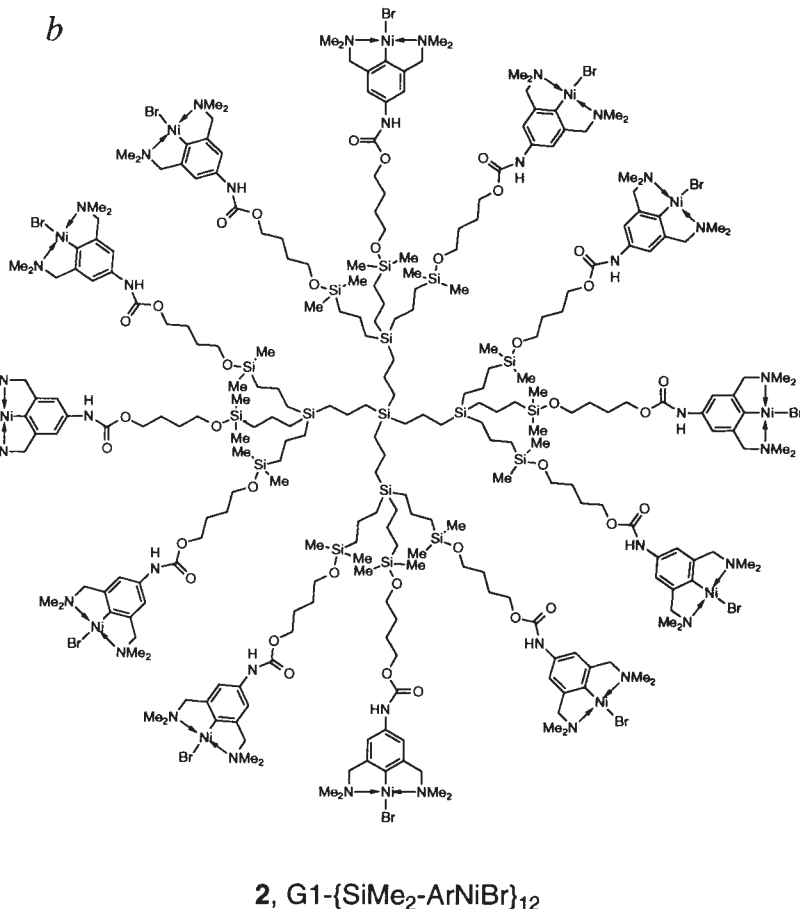
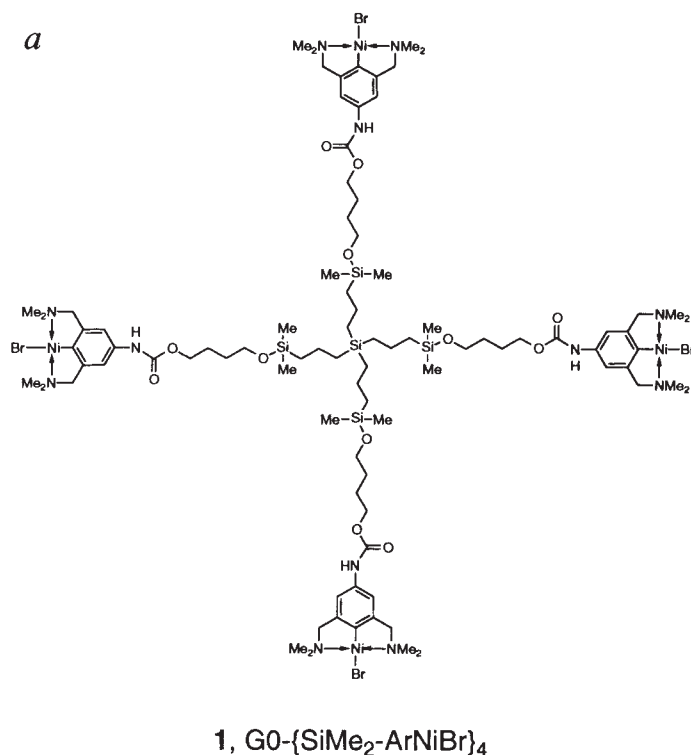


FIG. 1 Schematic structures of the functionalized dendrimer catalysts. *a*, **1**, G0- $\{\text{SiMe}_2\text{-ArNiBr}\}_4$; *b*, **2**, G1- $\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$.

G1) for which syntheses have been reported earlier by us^{13,14} and by others¹⁵. These silanes were converted with HSiClMe_2 to the corresponding SiMe_2Cl derivatives in which the number of Si-Cl functional groups is restricted to four ($\text{G0-}\{\text{SiMe}_2\text{Cl}\}_4$) and twelve ($\text{G1-}\{\text{SiMe}_2\text{Cl}\}_{12}$), respectively. In the next stage a diamino aryl bromide, the precursor entity for the organometallic site, was connected to the periphery of $\text{G0-}\{\text{SiMe}_2\text{Cl}\}_4$ and $\text{G1-}\{\text{SiMe}_2\text{Cl}\}_{12}$ by a 1,4-butanediol linker. To achieve this the 4-amino-substituted 2,6-bis[(dimethylamino)methyl]-1-bromobenzene, **4b** (ref. 2) was first reacted with triphosgene to afford the isocyanate derivative **4c** as its dihydrogenchloride salt¹⁶. This was subsequently converted with excess 1,4-butanediol (45 °C, 3 h) to the alcohol **4d**. Reaction of one mol-equivalent of $\text{G0-}\{\text{SiMe}_2\text{Cl}\}_4$ with four mol-equivalents of **4d** in the presence of triethylamine afforded the dendrimer system **5** ($\text{G0-}\{\text{SiMe}_2\text{-ArBr}\}_4$, Fig. 3) with a peripheral aryl bromide unit that was isolated in 81% yield after washing the reaction mixture with aqueous base and removing all volatiles *in vacuo*. In a similar way, starting from $\text{G1-}\{\text{SiMe}_2\text{Cl}\}_{12}$ and **4d**, the aryl bromide substituted dendrimer **6** ($\text{G1-}\{\text{SiMe}_2\text{-ArBr}\}_{12}$, Fig. 3) was obtained in 67% yield.

We found, based on earlier studies³, that dendrimer **5** reacts with excess zerovalent nickel complex $\text{Ni}(\text{PPh}_3)_4$ in tetrahydrofuran (THF) for 4 h at 60–70 °C to produce the desired oxidative addition product **1**, $\text{G0-}\{\text{SiMe}_2\text{-ArNiBr}\}_4$, (Fig. 1a). The crude product was separated from excess $\text{Ni}(\text{PPh}_3)_4$ and free PPh_3 by several precipitations from benzene and THF solutions with either hexane or ether to give **1** as an orange solid in 58% yield. In a similar way **2**, $\text{G1-}\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$ (Fig. 1b) was obtained from **6** ($\text{G1-}\{\text{SiMe}_2\text{-ArBr}\}_{12}$) in 60% yield.

The new metal-containing dendrimers **1** ($\text{G0-}\{\text{SiMe}_2\text{-ArNiBr}\}_4$) and **2** ($\text{G1-}\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$) have been unambiguously characterized by elemental microanalyses and ^1H and ^{13}C NMR spectroscopy. Like the mononuclear nickel complexes **3**, the functionalized dendrimers **1** and **2** both contain nickel(II) centres that are particularly sensitive to oxidation; the Ni(III) state, which is otherwise virtually unknown for organometallic species, is stabilized here by the special characteristics of the N,C,N'-coordinating diamino aryl ligand^{17,18} and it is this aspect that leads to catalytic activity^{19,20}. A practical consequence is that analytically pure samples of **1** and **2** give solution NMR spectra that tend to suffer from line-broadening when traces of paramagnetic Ni(III) centres are present; obtaining a ^{13}C NMR spectrum of the quality shown in Fig. 4 for complex **1** (CD_2Cl_2 , 75 MHz) requires particular care involving sample preparation under an inert (dinitrogen) atmosphere. An unexpected feature of this spectrum is that two sets of signals for the CH_2NMe_2 unit are resolved. Although the synthetic route is designed to produce metal sites with only Ni-Br functions, we are identifying here some sites with a Ni-Cl function; in separately prepared batches of **1** and **2**, generally ~20–30% of sites are Ni-Cl (determined by ^{13}C NMR). This phenomenon of chloride ion being carried forward in the synthetic scheme by the strongly coordinating amine functions of our dendrimer systems **5** and **6** is not detrimental in the current study; the Ni-Br and Ni-Cl functions in such arylnickel environments have very similar chemistry¹⁹. In CD_3OD , a polar solvent in which halide exchange occurs easily, the ^1H and ^{13}C NMR spectra of **1** give a single resonance pattern for the $-\text{C}_6\text{H}_2(\text{CH}_2\text{NMe}_2)_2-2,6$ groupings consistent with the presence of only one type of metal site.

Fast-atom-bombardment (FAB) mass spectrometric analysis of **1** (methanol solution added to a monothio glycerol or mNBA (*m*-nitrobenzyl alcohol) matrix affords spectra containing many ion clusters all having complex isotope patterns that, at this stage, are not suitable for assuring the fidelity of the proposed structure. We assume that the complexity results from the oxidation sensitivity of **1** in solution in combination with interactions with the matrices used and the presence of bromide and chloride in the sample. We are searching for the most appropriate procedures and techniques for acquiring representative mass spec-

TABLE 1 Catalytic activity of mononuclear complex **3a** and the dendrimers **1, 2**

Complex	Number of Ni sites (mol $\times 10^{-5}$)	Reaction time (h)	Conversion* (%)	Initial reaction rate† (turnovers per Ni site per h)
3a	10.06	0.25	21	234
	10.06	2	80	—
1 , $\text{G0-}\{\text{SiMe}_2\text{-ArNiBr}\}_4$	10.01	0.25	17	190
	10.01	2	74	—
2 , $\text{G1-}\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$	9.4	0.25	14	167
	9.4	2	63	—

Reaction conditions: to a solution of methyl methacrylate (2.8 g, 28.0 mmol), CCl_4 (10.0 ml, 104 mmol) and *n*-dodecane as internal gas chromatography (GC) standard (2.0 ml) in CH_2Cl_2 (12 ml) at 25 °C, a solution was added of catalytic complex (33.2 mg **3a**; 56.8 mg **1**; 55.2 mg **2**) in CH_2Cl_2 (2 ml).

* Selectivity for the formation of the 1:1 adduct of methyl methacrylate with CCl_4 is 100%. Conversion calculated from formation of the 1:1 adduct as determined by GC analysis.

† During the first 15 min.

tral data of multinuclear dendrimeric species **1** and **2**, as well as of mononuclear complexes **3**, so that in future research mass spectrometry can be used as a tool for identifying and characterizing such dendrimeric organometallic molecules.

The dendrimers **1** and **2** have been successfully employed as homogeneous catalysts for the Kharasch addition reaction. Under standard reaction conditions (room temperature, CH_2Cl_2 as solvent) using methyl methacrylate as substrate with CCl_4 as reagent, the catalytic activity of these two dendrimers as shown by kinetic data (Table 1) is 20% and 30% less, respectively, than that of the monomeric organometallic complex **3a** (initial rate ± 234 turnovers per metal site per hour). This similarity in reactivity magnitude was expected; molecular models of the two dendrimers show the nickel sites to be well separated from one another at the end of long flexible branches and consequently the accessibility of the catalytically active nickel centre should be similar to that in **3a**. In separate kinetic studies using derivatives of **3a** we have established that the electron-withdrawing *para*-substituent $-\text{NHC}(\text{O})\text{Oalkyl}$ has a small positive effect on the catalytic activity compared to H, (unsubstituted **3a**)². In the dendrimeric catalytic system all characteristics found for the monomeric model are retained. That is, there is clean regio-specific formation of the 1:1 addition product without polymerization of the alkene or production of C_2Cl_6 from CCl_3 radical dimerization.

Thus the new dendrimers **1** ($\text{G0-}\{\text{SiMe}_2\text{-ArNiBr}\}_4$) and **2** ($\text{G1-}\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$), represent nanoscopic catalysts with physical characteristics such as size, solubility and dispersity of catalytic sites that—unlike those in related systems with conventional polymers³—are very precisely defined, so affording these molecules advantageous properties for physical separation and catalyst recycling. The divergent synthesis employed for zero- and

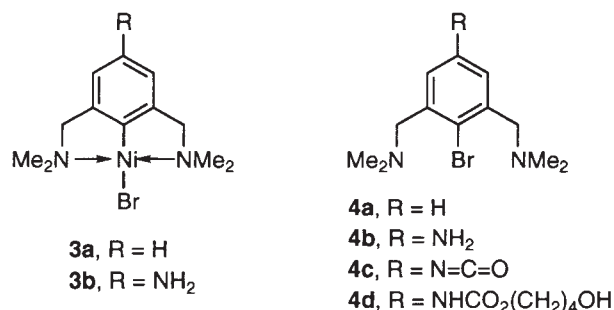


FIG. 2 Mononuclear arylnickel(II) complexes **3** and aryl bromides **4**.

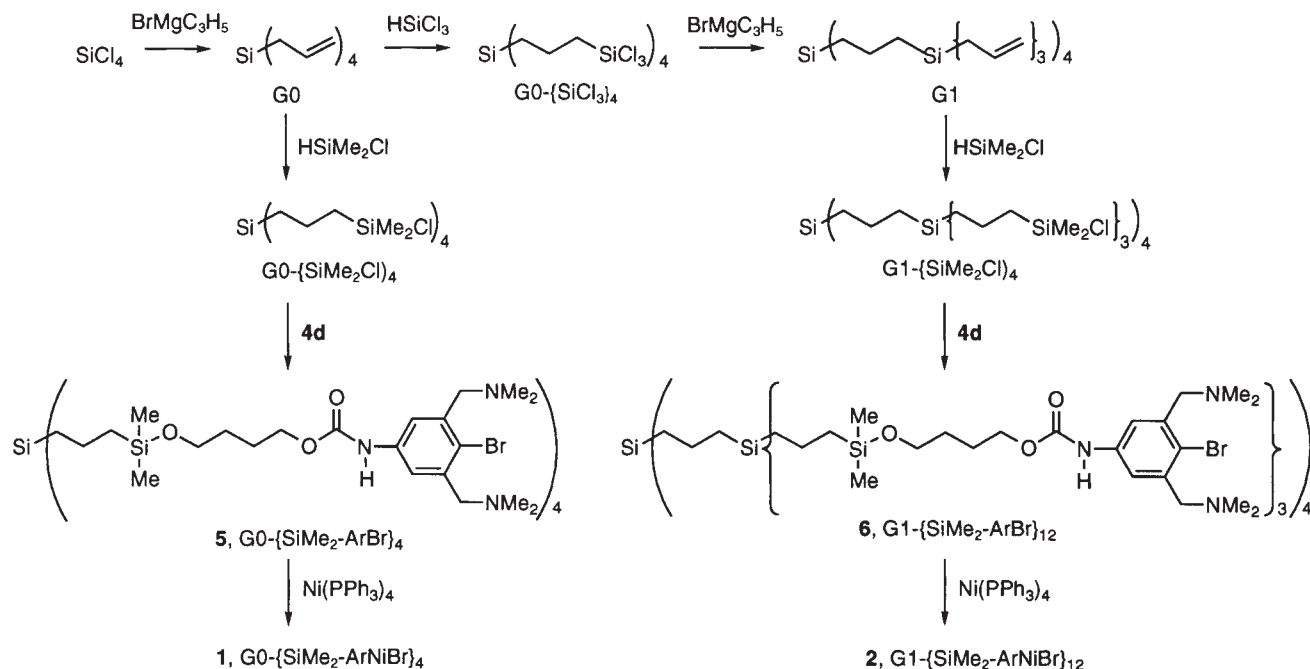
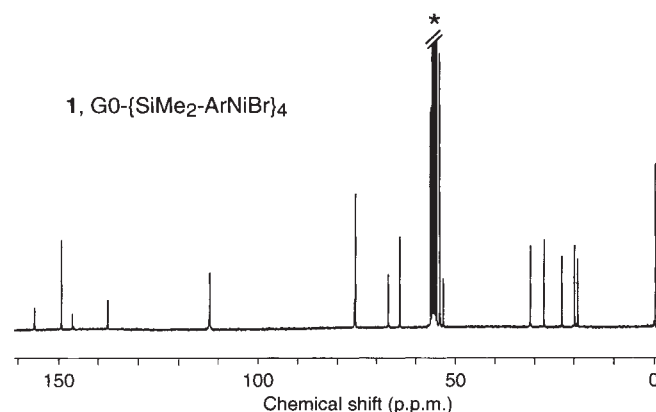


FIG. 3 Divergent synthesis of various functionalized silane dendrimers. Specific reaction conditions: **4d**, with CH_2Cl_2 , NEt_3 , 20 min reflux; $\text{Ni(PPh}_3)_4$, with THF, 60–70 °C, 4h. Selected physical data for **1**, $\text{G0-}\{\text{SiMe}_2\text{-ArNiBr}\}_4$: ^1H NMR (300 MHz, CD_2Cl_2): chemical shift δ 6.82 (br s, 4 H, NH), 6.71 (s, 8 H, aryl), 4.10 (t, $J=6.4$ Hz, 8 H, CO_2CH_2), 3.61 (s, 16 H, CH_2N), 3.59 (t, $J=6.0$ Hz, 8 H, CH_2OSi), 2.63 and 2.68 (2 $\times$ s, 48 H, NMe_2), 1.68 (m, 8 H, $\text{CH}_2\text{CH}_2\text{OSi}$), 1.57 (m, 8 H, $\text{CO}_2\text{CH}_2\text{CH}_2$), 1.39 (m, 8 H, SiCH_2CH_2), 0.66 (t, $J=8.1$ Hz, 8 H, $\text{OSi}(\text{Me})_2\text{CH}_2$), 0.60 (t, $J=8.1$ Hz, 8 H, $\text{Si}(\text{CH}_2)_4$), 0.07 (s, 24 H, $\text{OSi}(\text{CH}_3)_2$). ^{13}C NMR (75 MHz, CD_2Cl_2): δ 154.16 (4 C, NHCO_2), 147.03 (8 C, aryl), 144.75 (4 C, aryl), 136.07 (4 C, aryl), 110.01 (8 C, aryl), 73.67 and 73.97 (8 C, CH_2N), 65.24 (4 C, CO_2CH_2), 62.39 (4 C, CH_2OSi), 51.39 and 52.25 (16 C, NMe_2), 29.45 (4 C, $\text{CH}_2\text{CH}_2\text{OSi}$), 26.01 (4 C, $\text{CO}_2\text{CH}_2\text{CH}_2$), 21.52 (4 C, CH_2SiO), 18.28 (4 C, SiCH_2CH_2), 17.51 (4 C, Si (CH_2)₄), -1.94 (8 C, SiMe_2). (J , homo- or heteronuclear spin-spin coupling constant; s, singlet; t, triplet; m, multiplet and br, broad.) Infrared: ν_{max} 3,280 (NH), 1,724 (C=O), 1,530 (NH), 1,225 (C–O) cm^{-1} . Elemental microanalysis: calculated for $\text{C}_{88}\text{H}_{156}\text{Br}_4\text{N}_{12}\text{Ni}_4\text{O}_{12}\text{Si}_5$; C, 46.58; H, 6.93; N, 7.41 Ni,

10.34; found; C, 46.76; H, 7.20; N, 7.37 Ni, 10.40%. Selected physical data for **2**, $\text{G1-}\{\text{SiMe}_2\text{-ArNiBr}\}_{12}$: ^1H NMR (300 MHz, CD_2Cl_2): δ 6.98 (br s, 12 H, NH), 6.68 (s, 24 H, aryl), 4.10 (t, $J=6.3$ Hz, 24 H, CO_2CH_2), 3.66 (br s, 48 H, CH_2N), 3.59 (t, $J=6.0$ Hz, 24 H, CH_2OSi), 2.71 (s, 48 H, NMe_2), 1.67 (m, 24 H, $\text{CH}_2\text{CH}_2\text{OSi}$), 1.59 (m, 24 H, $\text{CO}_2\text{CH}_2\text{CH}_2$), 1.38 (m, 32 H, SiCH_2CH_2), 0.67 (t, $J=8.0$ Hz, 24 H, $\text{OSi}(\text{Me})_2\text{CH}_2$), 0.60 (t, $J=8.1$ Hz, 40 H, $\text{Si}(\text{CH}_2)_4$), 0.08 (s, 72 H, $\text{OSi}(\text{CH}_3)_2$). ^{13}C NMR (75 MHz, CD_2Cl_2): δ 154.29 (12 C, NHCO_2), 148.2 (br, 36 C, aryl), 136.3 (br, 12 C, aryl), 109.6 (24 C, aryl), 73.2 (br, 24 C, CH_2N), 65.20 (12 C, CO_2CH_2), 62.44 (12 C, CH_2OH), 52.35 (br, 48 C, NMe_2), 29.48 (12 C, $\text{CH}_2\text{CH}_2\text{OSi}$), 26.03 (12 C, $\text{CO}_2\text{CH}_2\text{CH}_2$), 21.57 (12 C, CH_2SiO), 19.06 (4 C, $\text{CH}_2\text{SiCH}_2\text{CH}_2\text{CH}_2\text{SiCH}_2$), 18.35 (12 C + 8 C, $\text{OSiCH}_2\text{CH}_2 + \text{CH}_2\text{SiCH}_2\text{CH}_2\text{CH}_2\text{SiCH}_2$), 17.59 (12 C, $\text{Si}(\text{CH}_2\text{CH}_2\text{CH}_2\text{Si})_4$), -1.78 (24 C, SiMe_2). Infrared: ν_{max} 3,280 (NH), 1,725 (C=O), 1,530 (NH), 1,225 (C–O) cm^{-1} . Elemental microanalysis: calculated for $\text{C}_{276}\text{H}_{492}\text{Br}_{12}\text{Ni}_{12}\text{N}_{36}\text{O}_{36}\text{Si}_{17}$; C, 47.15; H, 7.05; N, 7.17; Ni, 10.01; found; C, 47.08; H, 7.10; N, 7.12; Ni, 10.10%.

FIG. 4 75 MHz ^{13}C NMR spectrum of dendrimer **1** in CD_2Cl_2 at 300 K; solvent peaks indicated by asterisk.



first-generation aryl bromide dendrimers **5** and **6** is not complicated and these compounds are now being employed as starting materials for the synthesis of other metal-containing materials with preselected (catalytic) properties that may find application in two-phase or membrane reactors. □

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Climate correlations between Greenland and Antarctica during the past 100,000 years

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THE ice cores recovered from central Greenland by the GRIP^{1,2} and GISP2³ projects record 22 interstadial (warm) events during the part of the last glaciation spanning 20–105 kyr before present. The ice core from Vostok, east Antarctica, records nine interstadials during this period^{4,5}. Here we explore links between Greenland and Antarctic climate during the last glaciation using a high-resolution chronology derived by correlating oxygen isotope data for trapped O₂ in the GISP2 and Vostok cores. We find that interstadials occurred in east Antarctica whenever those in Greenland lasted longer than 2,000 years. Our results suggest that partial deglaciation and changes in ocean circulation are partly responsible for the climate teleconnection between Greenland and Antarctica. Ice older than 115 kyr in the GISP2 core shows rapid variations in the $\delta^{18}\text{O}$ of O₂ that have no counterpart in the Vostok record. The age-depth relationship, and thus the climate record, in this part of the GISP2 core appears to be significantly disturbed.

The oxygen isotope ratio ($\delta^{18}\text{O}$) of O₂ in the modern atmosphere ($\delta^{18}\text{O}_{\text{atm}}$) is ~23.5‰ heavier than mean ocean water⁶, mainly due to fractionation of the oxygen isotopes during photosynthesis, respiration and hydrological processes^{7–9}. (See Fig. 1 legend for a definition of $\delta^{18}\text{O}$.) The $\delta^{18}\text{O}$ of sea water ($\delta^{18}\text{O}_{\text{sw}}$) has varied with fluctuations in the volume of continental ice¹⁰. During the past 135 kyr, $\delta^{18}\text{O}_{\text{atm}}$ has followed $\delta^{18}\text{O}_{\text{sw}}$ variations because photosynthesis transmits variations in $\delta^{18}\text{O}_{\text{sw}}$ to O₂ in air^{11,12}. Although $\delta^{18}\text{O}$ of O₂ varies with time, it is constant throughout the atmosphere at any one time because the turnover

time of O₂ (~1.2 × 10³ yr)⁹ is much longer than the mixing time of the atmosphere (~1 yr). Hence $\delta^{18}\text{O}$ of O₂ can be used to intercorrelate ice cores, and to correlate ice cores with the deep-sea sediment oxygen-isotope stratigraphy^{11,12}.

Sowers *et al.*¹¹ measured $\delta^{18}\text{O}_{\text{atm}}$ in the Vostok ice core and used the results to establish a chronology for trapped gases in that ice core which is consistent with the SPECMAP chronology (Fig. 1). We present a new record of $\delta^{18}\text{O}_{\text{atm}}$ from the GISP2 core (based on the analysis of 201 samples between 100 and 3,050 m depth) and use the results to establish a chronology which is consistent with that of Vostok¹¹ as well as the deep-sea sediment record (Fig. 1). Because $\delta^{18}\text{O}_{\text{atm}}$ is relatively constant between 25 and 49 kyr ago, we consider the correlations between Vostok, SPECMAP and GISP2 to be inadequate during this period, and focus our discussion on ice which is older than 49 kyr.

We derive an age-depth relationship for the GISP2 core by correcting for the fact that gases are trapped 70–90 m below the surface, making the gas younger than the surrounding ice^{13,14}. Our ice-core chronologies are not necessarily the most accurate, but they provide the most precise relative ages for comparing GISP2 and Vostok climate records with one another as well as with deep-sea records in the range 50–105 kyr. We estimate the uncertainty in relative ice ages at GISP2 and Vostok as <3 kyr, half of which comes from uncertainties in correlating gas records and half from uncertainties in differences between gas and ice ages at Vostok. We also note that our absolute chronology is in excellent agreement with that derived for the GRIP core by Dansgaard *et al.*² based on a flow model and assigned ages for two depths in the core.

In Fig. 1, we plot δD of Vostok ice (δD_{ice})^{5,15} and $\delta^{18}\text{O}$ of GISP2 ice ($\delta^{18}\text{O}_{\text{ice}}$)³ against SPECMAP age. The isotopic composition of hydrogen and oxygen in precipitation reflect condensation temperatures, with heavier values corresponding to warmer temperatures. There are 22 distinct interstadial events recorded in the GISP2 (and GRIP) record between 20 and 105 kyr ago (refs 1–3). These well documented events range in duration from ~0.4 to 12 kyr (from first warming to final cooling). Eight of the nine long (>2 kyr from glacial baseline to glacial baseline) interstadial events at GISP2 can be clearly recognized in the Vostok record as increases in δD of at least 15‰, corresponding to warmings of 2 °C or more^{16,17}. The correlation of events 8 and 12 to Vostok events dated at 39 and 46 kyr is uncertain because the Vostok chronology is poorly defined in this period. Small warming events, largely buried in the noise at Vostok, may correspond to the shorter Greenland interstadials, but these events cannot be intercorrelated with confidence.

The nature of the correlated long interstadial events differs between Greenland and Antarctica. In Greenland, interstadial events (from cold baseline to cold baseline) are characterized by very rapid warming, moderate cooling during the interstadial, and then very rapid cooling at its end. At Vostok, related events are characterized by slow warmings and slow coolings. Interstadial events 21 and 23 appear to coincide between GISP2 and Vostok, but we cannot say whether Greenland or Antarctica warmed first. Other events may be in phase or out of phase given present resolution. We are impressed by the observations that climate change is more rapid, and events more numerous, in Greenland than in Antarctica. We infer that, during the last glacial period, interstadials began in the Northern Hemisphere and subsequently spread to Antarctica if they lasted long enough. However, this inference cannot yet be proven. Furthermore, the nature of interstadial forcing and response may vary from one event to the next.

A number of factors may be responsible for the interstadial events observed in Greenland and Antarctica, and their approximate synchrony. Insolation is the first factor, especially because orbital frequencies have been identified in both the Vostok and Greenland temperature records^{2,18}. Interstadials 21 and 23 fall within marine isotope substages 5a and 5c, respectively, and

may be linked (perhaps with a 3–5 kyr delay) to the maxima in summertime insolation at 60° N which occurred about 84 and 105 kyr ago¹⁵. Long interstadial events 8, 12, 14, and 16+17 occur during a period of moderate summertime insolation at 60° N which lasted from 35 to 59 kyr and may have favoured these episodes of warmer climate.

A second factor is sea level (or continental ice volume), which is associated with much of the variability observed in the Vostok temperature record¹⁹. Changes in sea level and ice volume could directly affect climate by destabilizing ice shelves, by changing albedo, and by causing changes in the production rates of warm, salty water in marginal basins (and hence oceanic meridional heat fluxes)²⁰. There is a remarkably strong connection between long interstadial events in Greenland and minima in $\delta^{18}\text{O}$ of benthic foraminifera in equatorial Pacific core V19–30 (Fig. 1), as previously implied by Bond *et al.*²¹.

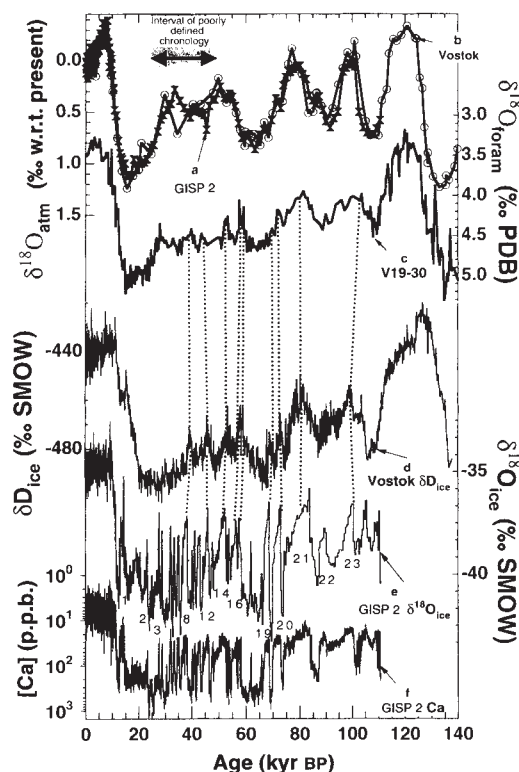
Third, North Atlantic Deep Water (NADW) production leads to a large flux of heat from oceans to atmosphere in the subpolar north Atlantic²². There is ample evidence that lower NADW production slowed or ceased during much of the last glacial period^{23,24}. Bond *et al.*²¹ and Keigwin and Jones²⁵ have invoked the startup of NADW production as the immediate cause of rapid warming associated with interstadial events in Greenland. North Atlantic Deep Water is an important source of warm, salty water to the Antarctic circumpolar current; it therefore contributes to oceanic heating of the atmosphere between 60° and 75° S (refs 26–28). Evidence for a link between NADW production and interstadial events at Vostok comes from recent work of Keigwin and Jones²⁵. They observed that changes in sedimentation in the western north Atlantic, thought to be induced by NADW formation, coincide with Vostok interstadials at ~50 kyr (corresponding to Greenland interstadial event

14), 55–60 kyr (events 16 and 17), 68 kyr (event 19), 72 kyr (event 20) and 80 kyr (event 21). Increased production of NADW from its low glacial values could have warmed Antarctica in two ways. (1) By increasing the heat released from the ocean to air^{26–29} causing a reduction in the maximum extent of winter sea ice which is further amplified by a reduced albedo, and (perhaps more likely) (2) by moving belts of Antarctic climate southwards and thereby increasing poleward heat transport through the atmosphere³⁰. This hypothesis is supported by Charles and Fairbanks' deep-water $\delta^{13}\text{C}$ record²³ from the Southern Ocean which suggests that the formation of NADW increased during the last deglaciation. On the other hand, modelling studies suggest that enhanced NADW production would actually cool the Southern Hemisphere by displacing warm intermediate waters towards the north^{31,32}. This latter work suggests that interstadial events in Greenland and Antarctica should be out of phase when they are unaccompanied by large variations in continental ice volume and atmospheric CO_2 . Given age uncertainties, we cannot exclude this possibility for related interstadial events 8, 12, 14, 16, 19 or 20.

The fourth event forcing factor is the greenhouse effect. During interstadial events, CH_4 concentrations rise by ≥ 100 parts per billion (by volume)^{33,34}, an increase which could cause temperatures at Vostok to rise by as much as 0.3 °C (~10% of the observed increase)³³. The link between CO_2 and Vostok temperatures is weaker. Only between 55 and 65 kyr ago (during interstadial events 16 and 17) does CO_2 increase coherently with Vostok temperature³⁵. The increase is ~25 parts per million (by volume), which could correspond to a temperature rise of up to 0.6 °C at Vostok, or ~20% of the total interstadial temperature rise.

FIG. 1 Greenland and Antarctic climate records covering the last glacial–interglacial cycle. For the timescale (kyr before present, BP), we adopt the layer-counting chronology of Meese *et al.*⁴⁵ and Alley *et al.*⁴⁶ for GISP2 to 2,250 m depth; we derive a gas age–depth relation for the GISP2 core between 2,250 and 2,800 m by correlating the GISP2 $\delta^{18}\text{O}_{\text{atm}}$ record into the Vostok $\delta^{18}\text{O}_{\text{atm}}$ record of Sowers *et al.*¹¹ between 37.9 and 110 kyr. a, b, $\delta^{18}\text{O}_{\text{atm}}$ records from GISP2 (a) and Vostok (b) ice cores. Note the interval over which the Vostok timescale is poorly defined (25–49 kyr). c, The benthic $\delta^{18}\text{O}_{\text{foram}}$ record from deep-sea sediment core V19–30 (3° 21' S, 83° 21' W, 3,091 m; *Uvigerina senticosus*)⁴⁷ showing continental ice volume variations. d, Vostok isotope temperature (δD_{ice}) with dashed lines tying the interstadial events at Vostok with the longer Dansgaard–Oeschger events in GISP2 ($\delta^{18}\text{O}_{\text{ice}}$) (e). Note the numbered interstadial events below the GISP2 record as identified in ref. 2. Also shown (f) is the [Ca] data from GISP2 on an inverted log scale. Our results indicate that for each Greenland interstadial event lasting >2 kyr, there is a corresponding warm event over Antarctica. Warm interstadial events may be related to local minima in the $\delta^{18}\text{O}_{\text{foram}}$ record from V19–30 indicative of high sea-level stands. Points on the ice age–depth curve for GISP2 lie as follows: 2,450 m, 52.4 kyr; 2,500 m, 57.5 kyr; 2,550 m, 63.8 kyr; 2,589 m, 69.5 kyr; 2,628 m, 75.6 kyr; 2,667 m, 82.2 kyr; 2,693 m, 87.6 kyr; 2,719 m, 93.8 kyr; 2,745 m, 100.1 kyr; 2,784 m, 107.4 kyr; and 2,808 m, 111.0 kyr. ($\delta^{18}\text{O} = \{[(^{18}\text{O}/^{16}\text{O})_{\text{sample}}/(^{18}\text{O}/^{16}\text{O})_{\text{standard}}] - 1\}$ per mil. The standard used is indicated on the figure: 'present', present-day values; PDB, standard rock; SMOW, standard mean ocean water. Similarly, $\delta D = \{[(D/H)_{\text{sample}}/(D/H)_{\text{standard}}] - 1\}$ per mil.)

METHODS. GISP2 samples (201) were analysed for $\delta^{18}\text{O}$ of O_2 and the $\delta^{15}\text{N}$ of N_2 using the method of Sowers *et al.*⁴⁸. We corrected the measured $\delta^{18}\text{O}$ of O_2 for gravitational fractionation using the measured $\delta^{15}\text{N}$ of N_2 in the following equation: $\delta^{18}\text{O}_{\text{atm}} = \delta^{18}\text{O}$ of $\text{O}_2 - 2(\delta^{15}\text{N}$ of $\text{N}_2)$. The standard deviation of $\delta^{18}\text{O}_{\text{atm}}$ values from the mean of replicates is $\pm 0.04\text{‰}$. The GISP2 $\delta^{18}\text{O}_{\text{atm}}$ record from 2,235.1 to 2,800 m depth was correlated into the Vostok $\delta^{18}\text{O}_{\text{atm}}$ record from Sowers *et al.*¹¹ between 37.9 and 110.0 kyr using an inverse correlation technique⁴⁹. The final mapping function has three coefficients and yielded an r^2 value of 0.96. We then constructed an ice age–depth profile by correcting for differences between the ages of gas and ice based on the protocol developed by Sowers *et al.*⁵⁰. The resulting ice age–depth profile for GISP2 differs from that based on layer counts



below 2,400 m (50 kyr). At this point the two age models begin to diverge with increasing depth until they reach a maximum difference of 26 kyr at 2,800 m. The origin of the discrepancy is being investigated. It may reflect a combination of loss of annual layers due to thinning and errors in the absolute chronology to which the $\delta^{18}\text{O}_{\text{atm}}$ curve is referenced.

Fifth and finally, ice-sheet modelling by MacAyeal suggests that some interstadial events may be caused by the episodic rapid discharge of large volumes of ice from the Northern Hemisphere ice sheets^{36,37}. Evidence of such discharge has been preserved in the Heinrich layers in North Atlantic ocean sediments^{21,38–40}. These Heinrich events have been closely correlated with the sequence of interstadial episodes in the Greenland ice core.

The slow change of Vostok temperatures during interstadial events suggests that the heat flux associated with changes in ocean circulation, as well as glacioeustatic changes in sea level, are important in transferring warmings in Northern Hemisphere climates to the Antarctic continent. Responses to other possible forcings are probably either too small (for example, radiative forcing by greenhouse gases) or too slow to account for the magnitude and shape of interstadial temperature–time curves observed at Vostok. Changes in atmospheric circulation could certainly be important, and could even have initiated the interstadial events, but would have been associated with changes in sea level or ocean circulation. With respect to climate variations induced by changes in ocean circulation, the time constant for reaching a new thermal steady-state might be as long as the oceanic turnover time of ~1 kyr. In the absence of ice-sheet surges, the time constant for growing or melting continental ice sheets is ~10 kyr. Melting ice sheets could cause sea level to rise a few tens of metres over a few thousand years. Thus partial deglaciation could also account for the response times of Antarctic climates.

In Fig. 2, we plot $\delta^{18}\text{O}_{\text{atm}}$ and $\delta^{18}\text{O}_{\text{ice}}$ records from the GISP2 core against depth for the interval of core below 2,750 m. We also plot Vostok δD and $\delta^{18}\text{O}_{\text{atm}}$ against age according to the extended glaciological time scale (EGT) of Jouzel *et al.*¹⁵ for the period from 100 to 220 kyr ago. It is immediately obvious from this figure that the GISP2 curve of $\delta^{18}\text{O}_{\text{atm}}$, so similar to Vostok above 2,790 m depth, is very different below 2,850 m depth. At Vostok, the depth interval containing ice deposited between 110 and 220 kyr ago is 1,600 to 2,500 m depth. This interval is 1,200–2,100 m above bedrock and therefore unlikely to have stratigraphic discontinuities. The lack of covariation in the $\delta^{18}\text{O}_{\text{atm}}$ records must, therefore, be due to disturbance in the GISP2 core below 2,790–2,850 m depth. Disturbance has, in fact, been observed in the fabric and stratigraphy of the GISP2 ice⁴¹ in the lower part of the core.

One interpretation of the GISP2 $\delta^{18}\text{O}_{\text{atm}}$ record below 2,850 m is that ice in the deep part of the core is affected by variable thinning but represents a continuous section except for minor loss of some parts of the section due to boudinage. According to this interpretation, $\delta^{18}\text{O}_{\text{atm}}$ minima at 2,846, 2,956 and 2,980 m depth can be correlated with minima at Vostok dated at 125, 180 and 210 kyr ago with the EGT chronology. The deepest $\delta^{18}\text{O}_{\text{atm}}$ minima in GISP2, at 2,998 and 3,002 m depth, are not found in the part of the Vostok core studied to date (back to 240 kyr) but might be correlative with the seawater $\delta^{18}\text{O}$ minima dated at 240 and perhaps 290 kyr ago in the SPEC-MAP timescale⁴².

Such a chronology for GISP2 is extremely unlikely to be correct, for the following reasons. First, it requires that there be much more high-frequency variability in the $\delta^{18}\text{O}_{\text{atm}}$ –time curve between 130–240 kyr than between 0–130 kyr. It also requires that this variability be absent from the Vostok record for the correlative period (age >130 kyr) because of a fortuitous choice of sampling depths in that core. Second, the above chronology requires that, between 175 and 185 kyr (corresponding to 2,950–2,960 m depth in GISP2), and during marine isotope stage 8 (corresponding to ~3,000 m depth in GISP2), Greenland temperatures be warmer than Holocene values, and that Ca concentrations reflect low interglacial values (indicating relatively wet climates and weak poleward atmospheric transport). Such conditions are not likely to have prevailed in Greenland throughout these periods when climates elsewhere were glacial^{15,43}. Third, the above chronology for GISP2 implies that Greenland tem-

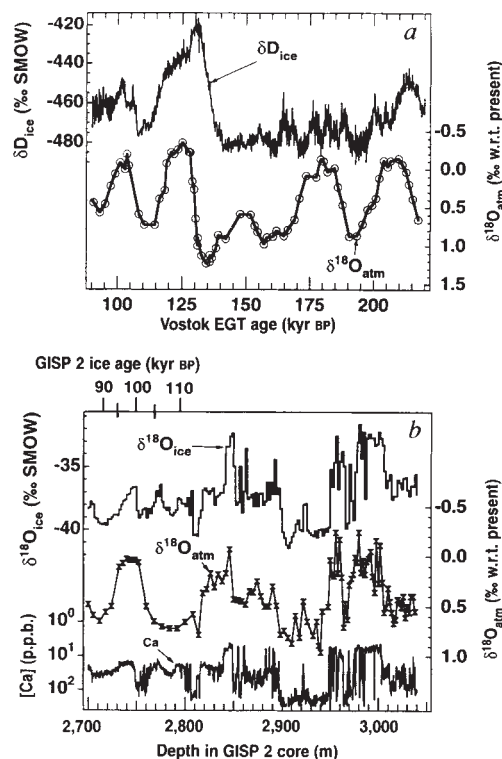


FIG. 2 a, δD of ice in the Vostok core for 90–240 kyr BP according to the EGT chronology¹⁶. Also shown in this panel is the Vostok $\delta^{18}\text{O}_{\text{atm}}$ record over the same time period showing smooth variations throughout. b, GISP2 $\delta^{18}\text{O}_{\text{ice}}$, $\delta^{18}\text{O}_{\text{atm}}$ and [Ca] records covering the bottom 350 m of the core. Of interest is the switch from smoothly varying $\delta^{18}\text{O}_{\text{atm}}$ above 2,800 m depth (100 kyr) to extreme $\delta^{18}\text{O}_{\text{atm}}$ variations below 2,800 m depth. Note the clear disagreement between the GISP2 and Vostok $\delta^{18}\text{O}_{\text{atm}}$ records in these two plots which we interpret as signifying discontinuities in the GISP2 core.

peratures during most of marine isotope stage 7 were warmer than Holocene and as warm as during substage 5e. This is possible but suspicious nonetheless: during stage 7 North Atlantic sea surface temperatures were lower, ice volume (as indicated by $\delta^{18}\text{O}$ of foraminifera) was greater, and Vostok temperatures were colder than during the Eemian²⁹.

An alternative explanation for the $\delta^{18}\text{O}_{\text{atm}}$ record in the deep part of the GISP2 core is that it reflects more significant disturbance of the original stratigraphy. This could happen in two ways. First, the section could still be ordered correctly in terms of age but could be highly condensed by extensive loss of ice due to thinning and boudinage. In this case, the ages given above would be minimum ages. Ca concentrations <10 p.p.b. and $\delta^{18}\text{O}_{\text{ice}}$ values >–34‰ indicate interglacial conditions; the deep part of the core could contain five or more interglacial periods and be well over 400 kyr old. Thus one could, for example, correlate GISP2 $\delta^{18}\text{O}_{\text{atm}}$ minima at 2,846 and 2,956 m depth with 125- and 210-kyr minima at Vostok, and GISP2 $\delta^{18}\text{O}_{\text{atm}}$ minima at 2,980 and 2,998–3,002 m depth with $\delta^{18}\text{O}_{\text{sw}}$ minima dated at 325 and 405 kyr in the SPEC-MAP timescale. The coldest part of stage 6, corresponding to the 180-kyr $\delta^{18}\text{O}_{\text{atm}}$ minimum at Vostok, and other parts of the climate record would be missing at GISP2 according to this scheme. Second, flow may have caused ice of different ages to be intermixed as a result of folding and/or intrusion⁴¹. In either case, rapid changes with depth in climate proxies could signify either rapid climate changes or disturbances in the age–depth relationship.

We note that the heaviest $\delta^{18}\text{O}_{\text{atm}}$ value observed below 2,750 m depth is $\sim\pm 0.9\%$. Comparison with the Vostok profile

shows that ice of age 130–140 kyr by the EGT chronology (corresponding to the coldest part of marine isotope stage 6) is therefore missing from GISP2, at least as sampled at 2–4 m depth increments. We also note that interglacial ice below 2,800 m in the GISP2 core consistently has $\delta^{18}\text{O}_{\text{atm}} = -0.25 \pm 0.10\%$, $[\text{Ca}] \sim 5$ p.p.b. and $\delta^{18}\text{O}_{\text{ice}} = -32 \pm 0.5\%$. We speculate that such ice samples may all have the same age, perhaps Eemian (corresponding to marine isotope substage 5e). It is possible that most ice in the GISP2 core below 2,850 m depth is largely from the latter part of Termination 2 (the penultimate deglaciation, ~ 128 –140 kyr BP) and substages 5e and 5d. Finally, comparison of our data with Vostok suggests that the ice is disturbed below 2,790 m but may largely be in correct stratigraphic order to 2,850 m depth. Thus, it may be that ice between 2,790 and 2,850 m depth corresponds to the latter part of the Eemian (substage 5e) and the subsequent cooling (5e/5d transition).

One of the most intriguing results to emerge from the recent study of the GRIP ice core was the rapid $\delta^{18}\text{O}_{\text{ice}}$ variations within the Eemian, which were interpreted as rapid changes to colder climates¹. This conclusion has been questioned by Taylor *et al.*⁴¹ on the grounds that the GRIP core, like GISP2, may be disturbed below 2,750 m (110 kyr BP). The GRIP core, drilled over the current ice divide, might have been subjected to less disturbance than GISP2 if the divide had remained at its present position. On the other hand, if the divide migrated, and Anandakrishnan *et al.*⁴⁴ have in fact argued that such migration is likely, then flow could have disturbed the GRIP stratigraphy just as we have observed at GISP2. The question of continuity of the “Eemian” and deeper sections of the GRIP ice core remains unresolved. However, this work demonstrates that continuity of the GRIP ice core below 2,750 m depth could be conclusively established by showing that $\delta^{18}\text{O}_{\text{atm}}$ varies as it does at Vostok. $\square$

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The role of deep roots in the hydrological and carbon cycles of Amazonian forests and pastures

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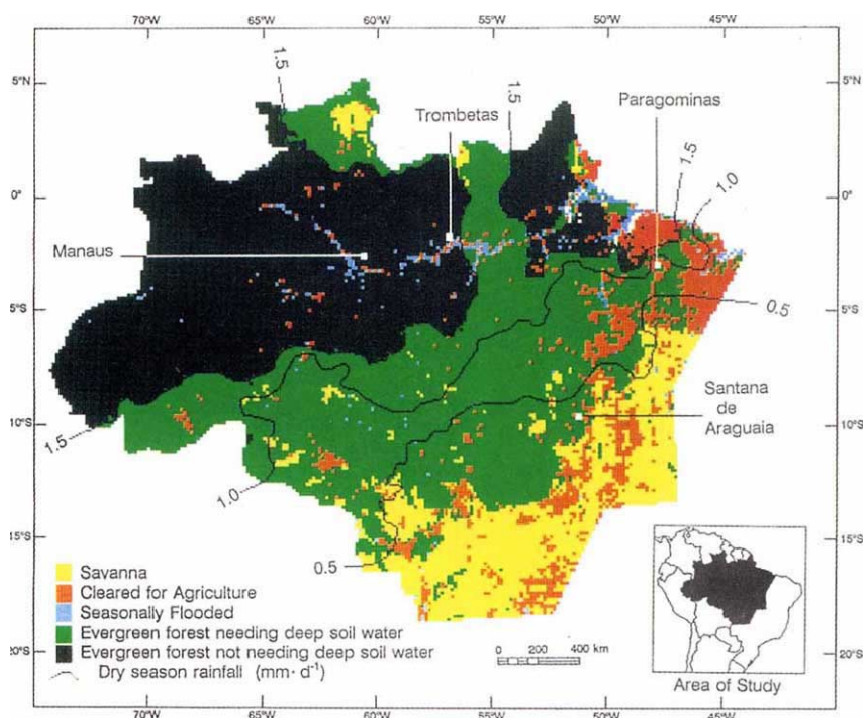
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DEFORESTATION and logging transform more forest in eastern and southern Amazonia than in any other region of the world^{1–3}. This forest alteration affects regional hydrology^{4–11} and the global carbon cycle^{12–14}, but current analyses of these effects neglect an important deep-soil link between the water and carbon cycles. Using rainfall data, satellite imagery and field studies, we estimate here that half of the closed forests of Brazilian Amazonia depend on deep root systems to maintain green canopies during the dry season. Evergreen forests in northeastern Pará state maintain evapotranspiration during five-month dry periods by absorbing water from the soil to depths of more than 8 m. In contrast, although the degraded pastures of this region also contain deep-rooted woody plants, most pasture plants substantially reduce their leaf canopy in response to seasonal drought, thus reducing dry-season evapotranspiration and increasing potential subsurface runoff relative to the forests they replace. Deep roots that extract water also provide carbon to the soil. The forest soil below 1 m depth contains more carbon than does above-ground biomass, and as much as 15% of this deep-soil carbon turns over on annual or decadal timescales. Thus, forest alteration that affects depth distributions of carbon inputs from roots may also affect net carbon storage in the soil.

Pastures are the most common type of vegetation on deforested land in Amazonia. They vary greatly in the ratio of grass to woody-plant cover, including managed pastures (from which woody vegetation is removed by heavy machinery) and the more common shrub- and tree-dominated “degraded” pastures^{15–17}. We studied deep roots in a mature, evergreen forest and in adjacent pastures near the town of Paragominas, in the Brazilian state of Pará. The deeply weathered clay soils at the

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FIG. 1 Major forest types and dry-season rainfall of Brazilian Amazonia. Savannas and deciduous forests (14%) were separated from evergreen forests (75%) based on seasonal patterns of canopy greenness (as seen from satellites) and a vegetation map. Evergreen forests include areas that did not display a seasonal depression of the normalized difference vegetation index (NDVI)^{28,29} during the dry seasons of 1986–88. To minimize the effects of clouds and smoke, only maximum monthly values of NDVI were used. Areas cleared for agriculture and seasonally flooded land (that is, pixels with the spectral signature of water) were adapted from Stone *et al.*²⁵. Dry-season rainfall refers to the driest three-month period of the year averaged for each rainfall record³⁰. Of the 212 weather stations used, 75% had more than eight years of rainfall data. Interpolation of rainfall data was conducted using a Geographic Information System software package (IDRISI; Clark Univ., Worcester, Massachusetts). Annual rainfall is 1,500 mm at Santana de Araguaia and daily rainfall is <0.5 mm during the driest three months. Annual rainfall at Manaus and Trombetas is 2,300 mm and daily rainfall is >1.5 mm during the driest three months. Evergreen forests in areas with <1.5 mm d⁻¹ during the driest three months of the year cover 36% of the region, and must depend on soil water extraction below 1 m depth, as described for the Paragominas study site (2° 59' S, 47° 31' W).



site are common in Amazonia¹⁸. Precipitation is highly seasonal with an average of 1,750 mm annually and <250 mm from July to November. This seasonality is typical of the eastern and southern portions of Amazonia where most deforestation is occurring (Fig. 1).

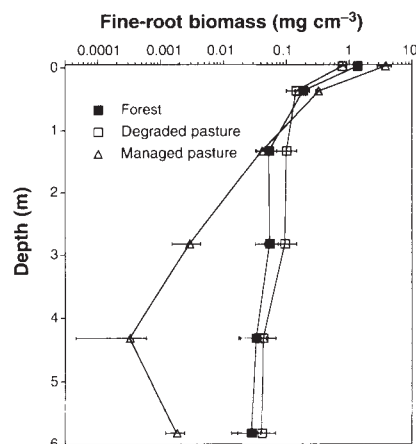
Roots were most abundant near the soil surface, as expected, but were found in deep shafts to depths of ~8 m in the managed pasture, ~12 m in the degraded pasture, and ~18 m in the forest. Fine-root biomass was lower in the top 10 cm of the degraded pasture soil than in the forest, but no differences occurred at greater depths. In the managed pasture, fine-root biomass was three times greater than in the forest near the soil surface and 15–100 times lower at depth (Fig. 2).

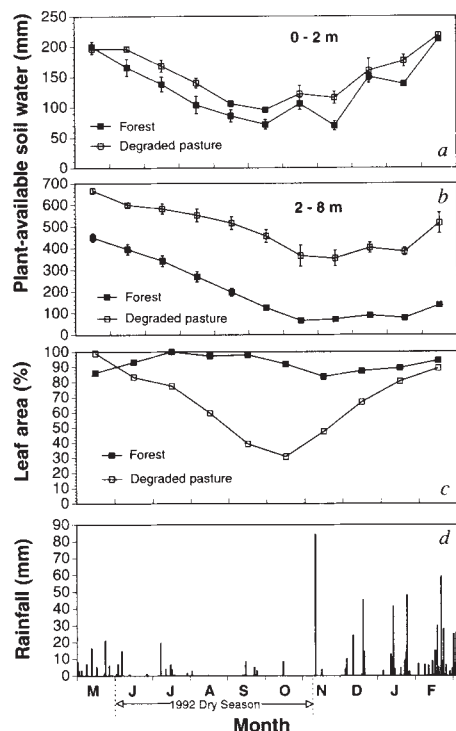
We assessed the hydrological role of deeply penetrating roots using a soil water-balance approach. During the severe 5.5-month dry season of 1992, when total rainfall was only 95 mm, plant-available soil water (PAW) from 2–8 m depth declined by 380 mm in the forest and 310 mm in the degraded pasture. In the upper 2 m of soil, the decline in PAW for this period was 130 mm and 100 mm, respectively (Fig. 3a, b). Hence PAW stored below 2 m in the soil provided >75% of the water

extracted from the soil in both ecosystems, and 100 mm more water was extracted from the forest soil. This is the case despite the fact that the deep forest soil began the 1992 dry season without being fully recharged (Fig. 3b) because of below-average rainfall in the wet season. More rainfall is needed to recharge the soil of the forest because it extracts more soil water during the dry season and because its canopy intercepts a larger portion of incoming rain.

Differences in water extraction between the forest and degraded pasture result in contrasting patterns of evapotranspiration. Evapotranspiration can be estimated for the dry season from the sum of rainfall and PAW depletion (surface runoff is insignificant in both ecosystems because of high soil infiltration rates¹⁹). During the dry season of 1992, average daily rainfall was 0.6 mm, and evapotranspiration in the forest and degraded pasture was 3.6 and 3.0 mm, respectively. Current vegetation-atmosphere models developed to predict the effect on climate of Amazonian deforestation assume rooting depths of only 1.3–2.0 m in forests and 0.6 m in pasture^{8–10} and, therefore, would underestimate evapotranspiration by >60% in these Paragominas ecosystems during the 1992 dry season. Important

FIG. 2 Vertical profile of live fine-root biomass (diameter <1 mm) in adjacent mature forest and man-made pastures near Paragominas, eastern Amazonia. Points are means, and bars are one standard error of the mean from 1.5-kg soil samples taken at each sampling depth in 36 auger borings in the forest, 17 in the degraded pastures, and 6 in the managed pasture. Roots were cleaned by flotation sieving and sorted manually at 10× magnification. The forest was cleared at the pasture sites in 1969, planted with the grasses *Panicum maximum* and later *Brachiaria humidicola*, and was heavily grazed intermittently to the present. Woody shrubs and treelets now comprise 50% of live leaf area in the degraded pastures and they support little grazing. The managed pasture was disk-harrowed, fertilized and replanted with *Brachiaria brizantha* in 1988, and is virtually free of woody vegetation.





hydrological processes, involving seasonal variation of deep water storage as high as 400 mm (Fig. 3b), are left out of models that ignore deep roots.

Evapotranspiration may have been reduced in the degraded pasture by a loss of green leaf area. The leaf area of degraded pasture vegetation declined 68% whereas average leaf area of forest plants declined only 16% during the 1992 dry season (Fig. 3c; the managed pasture lost 100% of its leaf area during this same period).

Less depletion of PAW in the degraded pasture signifies that this ecosystem can store less rainfall than the forest, and may therefore produce far more seepage to the groundwater aquifer or subsurface runoff to streams during the wet season. At the end of the 1992 dry season, the forest could store an additional

FIG. 3 Seasonal trends in soil water and leaf area in adjacent mature forest and degraded pasture, eastern Amazonia. Points are means, and bars are one standard error. *a*, Plant-available soil water (PAW) from 0–2 m depth. *b*, PAW from 2–8 m depth. *c*, Percentage of maximum leaf area of ten common species in the forest and degraded pasture, based on monthly observations of tagged branches ($n=20$ branches per species). In degraded pasture, values were weighted by the relative abundance of grasses and non-grasses. *d*, Daily rainfall. PAW was calculated as the amount of soil water held at tensions between -0.01 MPa (field capacity) and -1.5 MPa, determined from soil moisture retention curves for intact soil samples taken at various depths. Biweekly measurements of volumetric soil water content were made using time domain reflectometry, with sensors (6–8 per depth) installed at the surface and 1.5 m horizontally into the shaft walls at 1-m depth intervals^{31,32}.

770 mm of water in the upper 8 m of soil, compared to 400 mm in the pasture (Fig. 3a, b).

We assessed the role of root distributions in the soil carbon cycle by assessing soil C stocks and by analysing the isotopic composition of soil CO_2 and soil organic matter. The degraded pasture soil has lost 1.6 kg C m^{-2} in the top 1 m of soil relative to the forest (Fig. 4a); this loss is coincident with lower fine-root mass (and presumably lower C inputs) in the surface soil of the degraded pasture (Fig. 2). Changes in soil C stocks at depth are more difficult to determine reliably because small differences in C concentrations that are near detection limits must be extrapolated over large soil volumes. Soil C inventories and models of soil C processes seldom include analyses below 1 m, and the C present in deep soils is often assumed to be

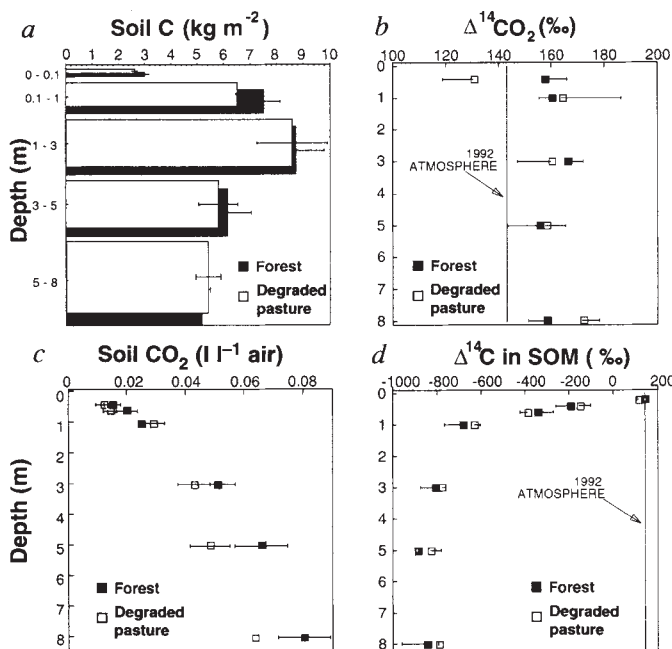


FIG. 4 Vertical profiles of soil C properties; points are means and bars are one standard error of the mean of three soil shafts per ecosystem. *a*, Soil C stocks (not including root C). *b*, $\Delta^{14}\text{CO}_2$ of the soil atmosphere. *c*, Concentration of CO_2 in the soil atmosphere. *d*, $\Delta^{14}\text{C}$ of soil organic matter (SOM) excluding root C. Soil gases were sampled through stainless-steel tubes (1.8 m long, 0.32 cm diameter) inserted horizontally into the sides of the soil shafts. Analysis of radon activities showed that the effect of gas exchange through the shaft wall was negligible. CO_2 concentrations were analysed in the field using a LiCor (Logan, Utah) infrared gas analyser. Gas samples were also stored in 0.5-l stainless-steel canisters for later analysis of $^{14}\text{CO}_2$ by accelerator mass spectrometry. Reported as $\Delta^{14}\text{C}$ (the $^{14}\text{C}/^{12}\text{C}$ ratio in parts per thousand corrected for variations in $^{13}\text{C}/^{12}\text{C}$ and compared to an absolute standard of oxalic acid in 1950), zero represents the 1950 atmospheric $^{14}\text{CO}_2$ content, positive values reflect the influence of ^{14}C derived from above-ground thermonuclear weapons testing, and negative values indicate that significant portions of the C have been isolated from exchange with atmospheric CO_2 long enough for the ^{14}C to decay (half-life, 5,730 years)²².

inert^{20,21}. But the soil C inventory below 1 m depth at Paragominas exceeds soil C above 1 m (Fig. 4a), as well as exceeding above-ground biomass (18 kg C m⁻²). Contrary to the assumption of inertness, our isotopic data indicate the presence of a significant fraction of modern C in deep soils. First, the $\Delta^{14}\text{C}$ (see Fig. 4 legend for definition of this quantity) below 1 m depth remains at or above modern atmospheric levels (Fig. 4b), indicating that the high concentrations of CO₂ observed at depth (Fig. 4c) result from root respiration and from microbial decay of carbon fixed by plants within the past 30 years²². Second, the $\Delta^{14}\text{C}$ of soil organic matter declines with depth, as expected, because the proportion of very old C increases with depth (Fig. 4d), but 10–15% of the soil C at 8 m depth may be modern (if the old C is essentially radiocarbon dead at a $\Delta^{14}\text{C}$ value of -1,000‰, the remaining modern fraction at +143‰ to +200‰ would be ~13% of the total C present at 8 m depth: $[(-850+1,000)/1,000]/[1+(143/1,000)] = 0.13$). This result suggests that up to 3 kg C m⁻² occurring below 1 m depth cycles on annual to decadal timescales, and that this C would be subject to change if root distributions were changed by land-use practices.

In the degraded pastures, woody plants have deep-root systems (Fig. 2) that may maintain the deep-soil C pool if fine-root turnover rates are similar to those in the forest. We have concentrated our studies of soil C dynamics on forests and degraded pastures, as these ecosystems are clearly the most abundant at present in eastern Amazonia. However, disk-harrowing and herd rotation can effectively exclude woody vegetation in more intensively managed pastures, and these practices are becoming more common^{16,17}. Based on root distribution (Fig. 2), we would predict that these managed pastures would lose deep-soil C and possibly gain surface-soil C (a hypothesis which we now intend to test). Differences in pasture management that affect distributions of C inputs throughout the soil profile may help explain why some studies have shown increases while others have shown decreases in C inventories of the surface soil following conversion of forest to pasture^{23–25}.

How common is deep rooting in the Amazon Basin? Roots have been inferred to 5 m depth in a forest of Surinam²⁷. Our shaft excavations to 8 m depth at forest sites that are less seasonal (Trombetas, Manaus) and more seasonal (Santana de Araguaia) than Paragominas (Fig. 1) revealed root distributions very similar to that of the Paragominas forest. These initial field data indicate that deep rooting is common in Amazonia.

We estimated the geographical distribution of deeply rooting forests in Brazilian Amazonia by overlaying monthly estimates of canopy greenness from AVHRR satellite imagery with a Geographic Information Systems database of rainfall from 212 weather stations. Where forests are evergreen but seasonal drought is significant (<1.5 mm d⁻¹ during the driest three months) we deduce that the forest must rely on water uptake from deep soil. The forests meeting these criteria cover an area of $\sim 1.8 \times 10^6$ km², which is most of the eastern and southern half of the Amazonian closed-canopy forest (Fig. 1). Hence deep roots play an important role in maintaining dry-season canopy greenness and evapotranspiration in the regions where human activity is concentrated. Deep roots are not limited to seasonally dry regions and may also play a role in nutrient uptake.

Deep roots help explain why Amazonian evergreen forests extend well into a region characterized by a long dry season. Understanding the effect of human land-use practices on regional budgets of water and carbon will require knowledge of the basic processes involving deep roots and deep soils. □

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Impaired recognition of emotion in facial expressions following bilateral damage to the human amygdala

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STUDIES in animals have shown that the amygdala receives highly processed visual input^{1,2}, contains neurons that respond selectively to faces³, and that it participates in emotion^{4,5} and social behaviour⁶. Although studies in epileptic patients support its role in emotion⁷, determination of the amygdala's function in humans has been hampered by the rarity of patients with selective amygdala lesions⁸. Here, with the help of one such rare patient, we report findings that suggest the human amygdala may be indispensable to: (1) recognize fear in facial expressions; (2) recognize multiple emotions in a single facial expression; but (3) is not required to recognize personal identity from faces. These results suggest that damage restricted to the amygdala causes very specific recognition impairments, and thus constrains the broad notion that the amygdala is involved in emotion.

We studied subject S.M., a 30-year old woman with normal IQ (low average; Wechsler Adult Intelligence Scale-Revised (WAIS-R) full scale = 86), a high-school education, and a neuropsychological profile remarkable for a history of defective personal and social decision making^{9,10}. On all occasions of testing, her mood was stable and cheerful, with no indication of depression on either observation or formal assessment (Beck Depression Inventory, Minnesota Multiphasic Personality Inventory). Her visual-perceptual discrimination assessed using unfamiliar faces was normal¹⁰. S.M. suffers from Urbach-Wiethe disease¹¹, a condition that caused a nearly complete bilateral destruction of the amygdala, while sparing hippocampus and all

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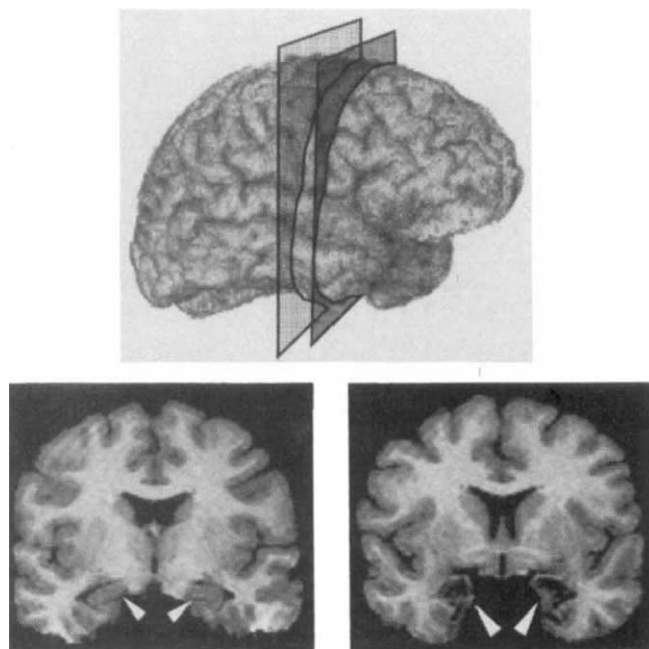


FIG. 1 t_1 -weighted MR images of S.M.'s brain. Planes of section are shown at the top, on a three-dimensional reconstruction²⁶ of S.M.'s brain. There is extensive bilateral amygdala damage (lower right image, large arrowheads) with sparing of neocortex and hippocampus (lower left image, small arrowheads). The tissue of the amygdala has been replaced by mineral deposits as a result of Urbach-Wiethe disease¹¹.

neocortical structures, as revealed by detailed neuroanatomical analyses of her computed tomography (CT)¹⁰ and magnetic resonance imaging (MRI)⁹ scans (Fig. 1).

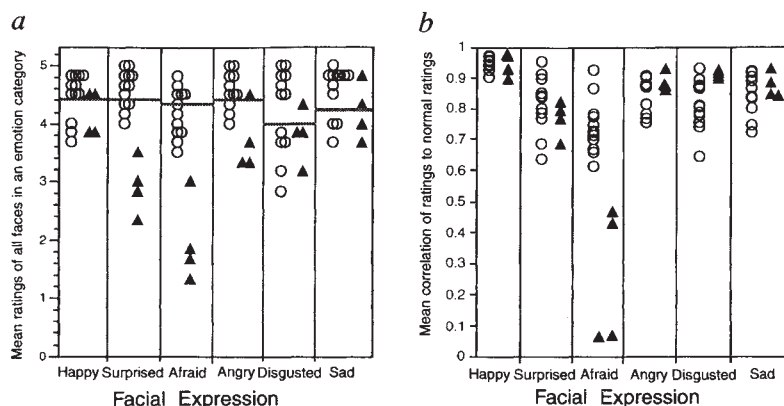
The human face conveys information about a person's identity, expresses emotion, and often signals blends of different

emotions at the same time, all of which are elements critical to social behaviour. We therefore used ecologically relevant stimuli (human facial expressions of emotion¹²) to test the hypotheses that bilateral destruction of the amygdala impairs (1) recognition of some basic emotional facial expressions, and (2) recognition of the combination of several emotions shown in a single expression. We compared S.M. to 12 brain-damaged controls of similar IQ (mean WAIS-R full-scale score = 95 ± 9 ; see Fig. 2 Methods for details). Subjects were shown facial expressions of six basic emotions (happiness, surprise, fear, anger, disgust, sadness), as well as neutral faces, and asked to rate each face according to several emotional adjectives. S.M. differed dramatically from controls in this task. She rated expressions of fear, anger and surprise as less intense than did any of the brain-damaged controls (Fig. 2a; mean ratings for S.M. were >3 s.d. below control mean for these emotions). We next assessed S.M.'s recognition by correlating her ratings of facial expressions with the mean ratings given by seven normal controls with no history of brain damage. S.M. showed a severe recognition impairment specific to fear: her ratings of fearful faces correlated less with normal ratings than did those of any brain-damaged control (Fig. 2b), and fell 2–5 s.d. below the control mean when the data were converted to a normal distribution. By contrast, S.M.'s recognition of the unique identity of faces was fully preserved: she correctly identified without difficulty 19/19 photographs of familiar faces, some of whom she had not seen for many years, and she easily learned to recognize new faces. These data provide evidence for a double dissociation between processing of facial identity and of facial affect, suggesting that the two are subserved by anatomically separable neural systems^{13–16}. Insofar as expressions of a single, basic emotion are concerned, the human amygdala's role appears to be quite specific to recognition of fear. This finding may parallel results from animal studies, which show that amygdectomy results in abnormal behaviour to frightening stimuli^{17–19}.

To explore in greater detail S.M.'s abnormal processing of facial expressions, we examined her ability to recognize similarities between different expressions. We analysed S.M.'s ratings

FIG. 2 Ratings of emotional facial expressions on emotional adjectives. *a*, Ratings of the intensity of an emotion expressed by typical expressions of that emotion. Rating scores on the emotional word for which the face was a typical example are shown as the mean of all faces within an emotion category. Data are from 12 brain-damaged controls ($\circ$), and from 4 experiments with S.M. ($\blacktriangle$). Mean ratings given by 7 normal controls (with no history of brain damage) are denoted by the dotted lines. *b*, Correlations of ratings of facial expressions with the ratings given by normal controls. Mean Pearson correlations are shown of mean normal control ratings ($n=7$) correlated with the ratings given by brain-damaged controls ($\circ$; $n=12$) and with S.M.'s ratings ($\blacktriangle$; 4 experiments). All normal controls' ratings correlated well with one another ($r>0.7$ for every emotion category).

METHODS. Thirty-nine expressions of facial affect from Ekman¹² were presented nine times in two blocks separated by several hours. Six faces (both male and female) each of anger, fear, happiness, surprise, sadness, disgust, and three neutral faces, were projected on a screen, one at a time. Subjects judged each face on a scale of 0–5 (0 = not at all; 5 = very much) with respect to 9 adjectives: happy, sad, disgusted, angry, afraid, surprised, awake, sleepy, interested (1 adjective per trial). All data were used in Fig. 3 to obtain good MDS solutions; ratings on only the 6 most consistently rated adjectives (excluding the non-emotional words 'awake', 'sleepy', 'interested') were used in all other figures. Independent tasks showed that all subjects understood the adjectives. Correlations of a subject's ratings with normal controls' ratings were calculated for each facial expression, Z-transformed to normalize their distribution, averaged over faces that expressed the same emotion, and inverse Z-transformed to give the mean correlation for that emotion category. S.M. repeated the



experiment 4 times, twice on 2 days separated by 6 months, with 2 experimenters who were blind to the nature of the experiment. Control subjects: all brain-damaged control subjects were selected from our patient registry and had been fully characterized neuroanatomically and neuropsychologically²⁷. MRI or CT scans of each subject revealed no sign of damage to the amygdala. We excluded subjects who had emotional dysfunction (such as depression). To reduce a possible effect of task difficulty, our 12 controls included subjects with IQ lower than that of the target subject (S.M.). We took into account visual complexity of the stimuli and the possibly abstract nature of the task by including control subjects with impairments of vision (occipital lesions; $n=6$) and executive functions (frontal lesions; $n=3$). Five of the controls had bilateral lesions. All subjects used in our study had given informed consent to participate in research.

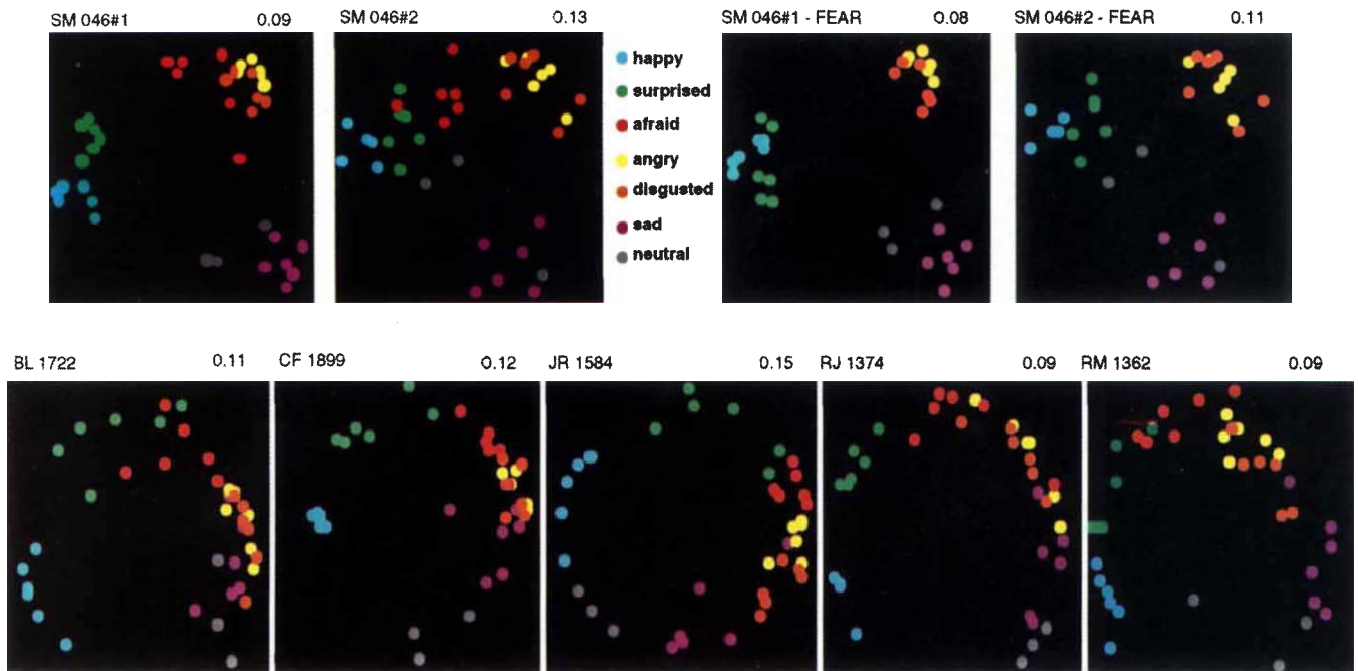
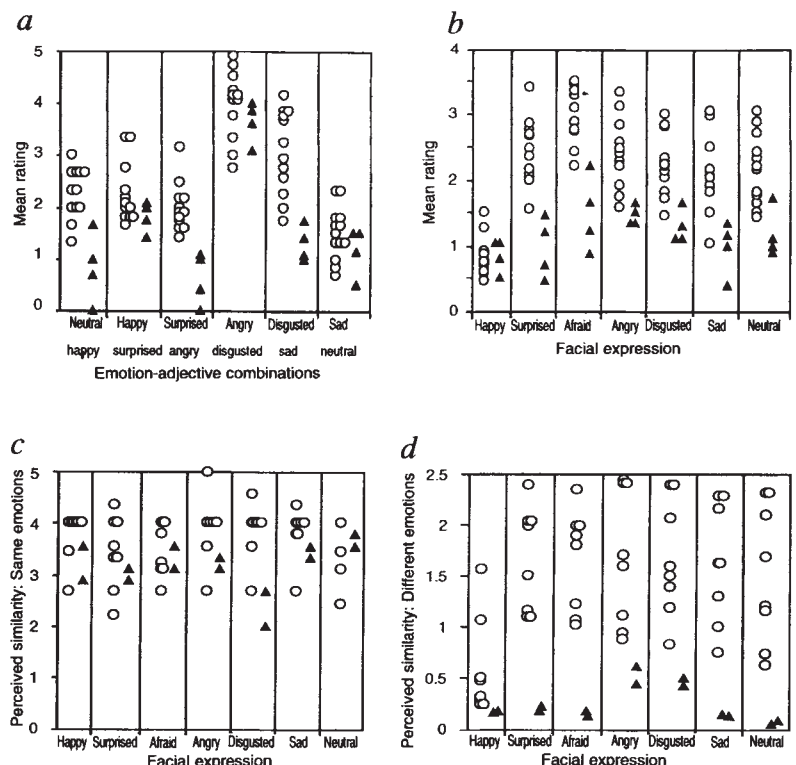


FIG. 3 Multidimensional scaling (MDS) of perceived similarity judgments of emotions expressed by faces. Each coloured point represents one of the 39 facial expressions which subjects had to rate. Euclidean distance between points corresponds to perceived dissimilarity between stimuli. Small numbers in upper left are the subject's code; to the right is the MDS stress, a measure of 'good-ness-of-fit' (smaller numbers are better fits). Top, MDS plots from 4 experiments with S.M. Each plot uses non-overlapping data from 2 different experiments. The left 2 plots show MDS on all the faces; the right 2 plots show MDS on all faces except fear. The similar structure of the left and right plots suggests that impaired recognition of fear alone does not account for the clustering seen. Bottom, Representative plots from 5 of the brain-damaged controls. We examined the data from all 12 controls, and also scaled all

data with fearful faces subtracted; in every case we obtained circular arrangements. No control showed clusters like S.M., even when MDS stress was high.

METHODS. For each subject, ratings from 2 experiments were summed. Rating profiles for each of the 39 expressions were correlated with one another. This correlation matrix was used to compute non-metric MDS of dissimilarities in Euclidean 2-space with two algorithms^{28,29} that yielded identical results. Similarity varied with Euclidean distance as a smooth, nearly linear, monotonically decreasing function. We show MDS solutions only for a stress ≤ 0.15 , corresponding to an RSQ > 0.9 (RSQ is a measure of the proportion of the variance in the data accounted for by the MDS solution).

FIG. 4 Recognition of multiple emotions in facial expressions. **a**, Mean ratings of facial expressions on adjectives denoting emotions adjacent in the MDS plots of Fig. 3. The emotion-adjective combinations are shown on the x-axis; data were averaged over 6 faces within each emotion category except neutral (3 faces), and over the 2 cases where emotional expression and adjective are interchanged. S.M.'s (Δ , 4 experiments) low ratings compared to the controls' ratings ($\circ$, $n=12$) correspond to the gaps in her MDS plots. **b**, Mean ratings of all the emotions other than the prototypical emotion recognized in an expression. S.M. tends not to endorse any emotions other than the prototypical ones. **c**, Direct similarity ratings of faces expressing the same emotion. **d**, Direct similarity ratings between faces expressing different emotions. Each category shows average similarity ratings of all 54 possible pairings of expressions (3 expressions of the given emotion $\times$ 6 other emotions $\times$ 3 expressions). S.M. fails to recognize similarity between expressions of different emotions (number of s.d. different from control mean: happy = 0.8, surprised = 2.8, afraid = 3, angry = 1.6, disgusted = 2.1, sad = 1.9, neutral = 2.5). **METHODS.** **c**, **d**, Subjects directly judged similarity of emotion expressed by 21 of the faces used in all other experiments (3 of each of the 7 emotion categories) on a 5-point scale. All possible pairwise combinations (231) of facial expressions were presented as adjacent photographs, a pair at a time. Care was taken to ensure the subject understood both the rating scale and rated similarity of emotion. Data are from 8 of the brain-damaged controls, and from 2 experiments with S.M. All controls whose MDS is shown in Fig. 3 are shown here.



of emotional faces with a multidimensional scaling (MDS) technique, in which the perceived similarity among expressions corresponds to their proximity in the scaled representation (Fig. 3). For example, surprised and happy faces are rated similarly, and hence are adjacent in MDS plots, but happy and sad faces are very different, and hence far apart. The arrangement of emotions in the MDS plots of our controls (Fig. 3) is similar to what has been published for normal subjects, for judgements of emotional facial expressions²⁰ and emotional words²¹ obtained in several different cultures²². This arrangement suggests that facial expressions have graded membership in categories of emotion, and that an expression can be a member of more than one emotion category. By contrast, S.M. perceived emotions more discretely, and did not generate the nearly continuous circular ordering that the controls showed. To ensure that S.M.'s abnormal MDS arrangement was not simply a consequence of her impaired recognition of fear, we also present the MDS solutions obtained without fearful faces (Fig. 3). The results are very similar. We also obtained similar results when we scaled stimuli on all the adjectives excluding 'afraid'. S.M.'s inability to perceive similarity between expressions of different emotions thus appears to be independent of her impaired recognition of fear, although the two may nonetheless be consequences of a single processing dysfunction.

Does S.M. fail to recognize similarities between expressions of different emotions because she cannot recognize the blend of multiple emotions conveyed by a single face? A quantitative analysis of S.M.'s inability to recognize multiple emotions in a facial expression is given in Fig. 4. The gaps in her MDS plots (Fig. 3) correspond to the endorsement of very low ratings when judging expressions of different emotions on each other's adjectives (for example, how much disgust is there in sad expressions, and vice versa?) (Fig. 4a). S.M. showed a tendency to recognize only the prototypical emotion in a facial expression, with the exception of some expressions that subjects all judge to be very similar, such as anger and disgust (Fig. 4a, b). This finding was replicated with a task that relies less on language. We asked subjects to rate each of 231 possible pairings of 21 photographs of facial expressions (3 of each emotion) in terms of similarity of the emotion expressed. S.M. appropriately judged all the expressions of the same emotion to be similar to one another, but she failed to recognize similarity among expressions of different emotions (Fig. 4c, d).

Facial expressions can convey both basic emotions whose expression and recognition may be partly innate^{23,24}, as well as subtler emotions whose meaning is partially determined by culture²⁵. From our results, the amygdala appears necessary both to recognize the basic emotion of fear in facial expressions, and to recognize many of the blends of multiple emotions that the human face can signal. The amygdala may be an important component of the neural systems subserving social cognition in part because fine-grained recognition of the emotions signalled by faces is essential for successful behaviour in a complex social environment. □

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Mutations in the *DAX-1* gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism

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ADRENAL hypoplasia congenita (AHC) is an X-linked disorder characterized by primary adrenal insufficiency^{1,2}. Hypogonadotropic hypogonadism (HHG) is frequently associated with this disorder but is thought not to be caused by the low adrenal androgen levels due to adrenal hypoplasia^{3,4}. It is uncertain whether there are two distinct yet physically linked genes responsible for AHC and HHG or a single gene responsible for both diseases. AHC can occur as a part of a contiguous deletion syndrome together with Duchenne muscular dystrophy (DMD) and/or glycerol kinase deficiency (GKD). From the analysis of deletions, the following gene order has been deduced: Xpter-AHC-GKD-DMD-cen^{5,6}. An

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AHC critical region of 200–500 kilobases has been defined by physical mapping^{7,8} and partially overlaps with a 160-kilobase dosage-sensitive sex (DSS) reversal critical region⁹. The *DAX-1* (DSS-AHC critical region on the X, gene 1) gene was isolated and found to encode a new member of the nuclear hormone receptor family¹⁰. Here we report that *DAX-1* is deleted in 14 patients and point mutations were found in the coding region in DNA from 12 unrelated individuals. All AHC patients over 14 years old and with only point mutations in *DAX-1* were also diagnosed with HHG, confirming that the *DAX-1* gene is responsible for both X-linked AHC and HHG. But in four sporadic cases and a single familial

case, no point mutations were found, suggesting genetic heterogeneity or differential expression of *DAX-1*.

To understand non-overlapping deletions and complex rearrangements described in several AHC patients^{7,8}, we constructed a detailed map of the AHC/HHG critical region defined by deletions (Fig. 1 and Table 1). We localized *DAX-1* (a two-exon gene spanning 5 kilobases (kb) of genomic DNA¹⁰) and all known markers across the AHC critical region on our yeast artificial chromosomes (YAC) and cosmid contiguous units (contigs). *DAX-1* was localized in cosmid contig A, 60 kb from QST59 (Fig. 1). We integrated two previously reported maps of

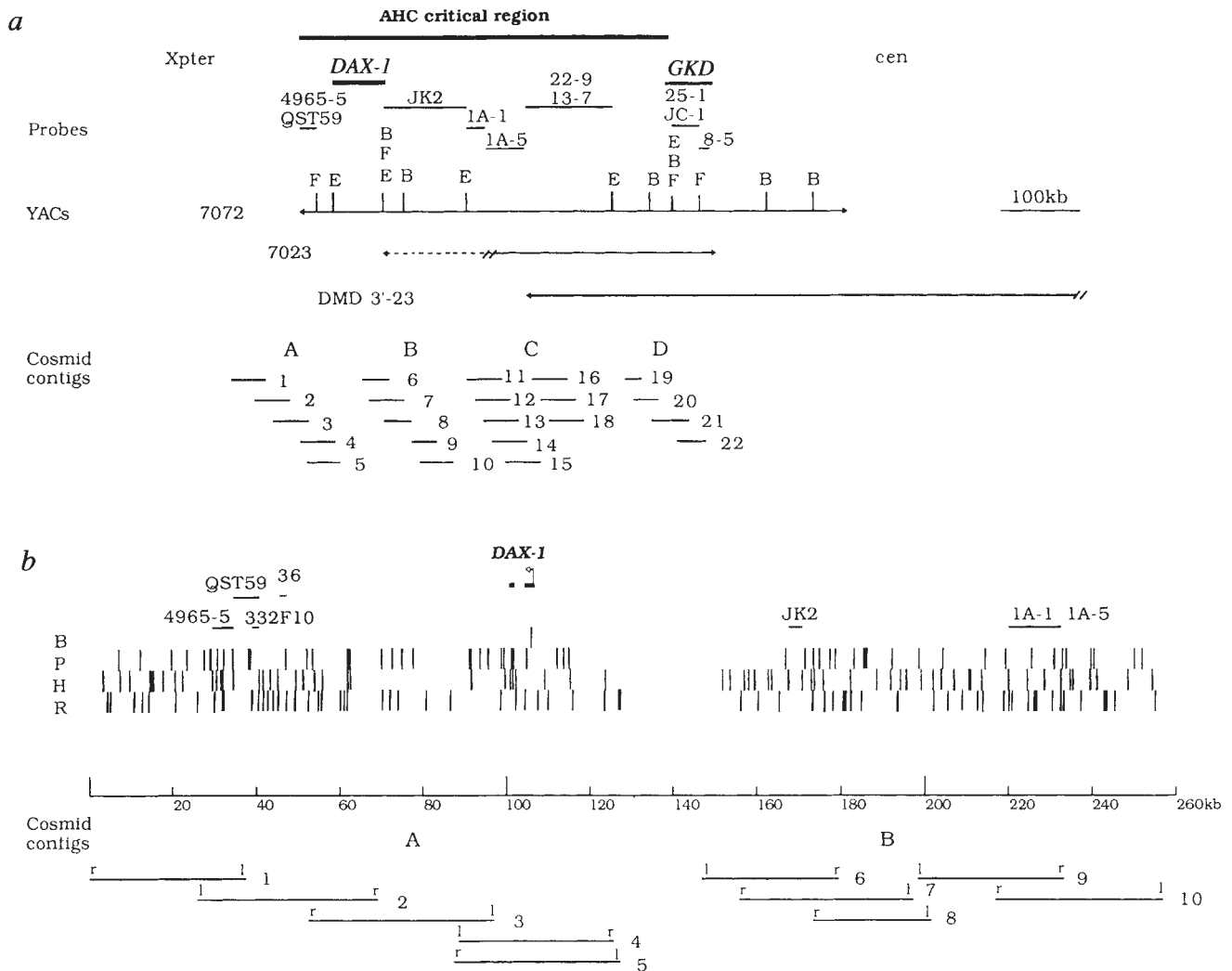


FIG. 1 Physical mapping of the AHC-HHG critical region. **a**, The YAC contig was mapped by partial digestion with *Sfi* (F); *Bss*III (B); and *Eag*I (E). A double slash across a YAC indicates the position of a chimeric junction. The cosmid contigs A–D are indicated by a minimum set of clones, which were mostly obtained by subcloning YAC 7072 and are available on request. Contig A, of about 125 kb, encompasses the probes 4965-5 (DXS1074), 332F10 (DXS1020) and QST59 (DXS319). Contig B, of 110 kb, encompasses the probes JK2 (DXS1023), K23b (DXS1095), 1A-1 and 1A-5 (DXS1075). Contig C, of about 200 kb, encompasses the probes 22-9 and 13-7 (DXS1076) and contig D, of about 100 kb, encompasses the GKD locus and the probes 25-1 (DXS1077), JC-1 (DXS708) and 8-5 (DXS1078). **b**, A high-resolution map of cosmid contigs A and B in the AHC–HHG critical region. Cosmids were mapped by partial digestion with *Bss*III (B); *Pst*I (P); *Hind*III (H); and *Eco*RI (R). Genes and probes are mapped with respect to this

restriction site map. The transcriptional orientation of *DAX-1* is 5'–3' from centromere to telomere, as shown by the arrow. The two bars show the position of the two *DAX-1* exons. Cosmids are numbered as in **a**. l, Left arm, r, right arm. For cosmids 3–5, *Hind*III sites were incompletely detected. The gap between contigs A and B was estimated at 20 kb by fluorescent *in situ* hybridization on released chromatin preparations (G. Senger, personal communication) and physical mapping data.

METHODS. Cosmids were obtained from the ICRF flow-sorted X chromosome reference library¹⁸ or from a library subcloned from YAC 7072 (ICRFy900A0269) into SuperCos vector using the manufacturers' protocol (Stratagene). The cosmids were ordered by hybridization both with previously mapped probes, and with cosmid end riboprobes. Partial restriction digestion was as described^{19–22}.

the AHC region^{7,8} allowing accurate estimates of the size of AHC deletions and the location of breakpoints.

The *DAX-1* gene was found deleted in 14 unrelated patients listed in Table 1 and a new double deletion in patient M.C. was also detected, a phenomenon previously noted in the AHC-GKD patients B.E.N.⁷ and Y.B.⁸. The smallest deletion detected (patients 3969 and 4445) is about 10 kb including the first exon and promoter region of *DAX-1*.

Seventeen unrelated patients without deletion or rearrangement detectable by Southern blotting were analysed for point mutations by direct sequence analysis of polymerase chain reaction (PCR) products derived from genomic DNA¹¹ (Fig. 2*Aa-c*). Mutations in *DAX-1* were found in 11 AHC families and one sporadic case (summarized in Table 2). Patient M.A. (family 1) showed a complex deletion-insertion (Fig. 2*Ad*) which could have resulted from a non-homologous recombination mechanism between the two alleles.

In summary, five nonsense mutations, five frame-shift mutations, one missense mutation (R267P) and one deletion of a single amino acid (dV269) have been detected in the unrelated patients. Localization of all the mutations in the *DAX-1* protein is shown in Fig. 2*B*. From both deletion and mutation studies, we confirm that *DAX-1* is involved in X-linked AHC. The nonsense and frameshift mutations could result in a non-functional truncated protein or decreased RNA stability, resulting in the absence of any form of protein¹². The two mutations changing single amino-acid positions (positions 267 and 269) are located within the putative ligand-binding domain in a region of high sequence conservation. Both changes are predicted to result in structural changes of the *DAX-1* protein (DAX-1) and the complete AHC-HHG phenotype of the patients suggests that this region is indeed essential for *DAX-1* function. Until the three-dimensional structure of the ligand-binding domain of nuclear receptors is known, point mutations provide the only clue as to the functional importance of individual amino-acid residues.

There is an intra-familial variability in the age of onset from a few days after birth to 10 years (families 10, 11, 12). In accordance with this observation, there appears to be no correlation between the type of mutation and the age of onset of the disease.

No point mutations in *DAX-1* were found in either the coding region or 5' and 3' splice sites in five AHC patients: four are sporadic and one is familial (Table 2). These patients have symptoms of adrenal insufficiency typical of AHC and, in the familial case, the cytomegalic form of AHC was histologically confirmed, suggesting X-linked inheritance in this family (data not shown). Three patients were analysed further for the integrity of the *DAX-1* transcript. Reverse-transcribed PCR was done using poly(A)⁺ RNA extracted from lymphoblastoid cell lines from a normal individual and patients B.A., B.V. and 2703. Two rounds of PCR using nested primers produced a visible PCR product of the same size both in normal controls and in patients (data not shown). This result rules out the possibility that the intron of *DAX-1* is disrupted resulting in abnormal splicing; such a phenomenon has been observed in the factor VIII gene^{13,14}. *DAX-1* can be transcribed as an illegitimate transcript¹⁵ in the lymphocytes of patient B.A., B.V. and 2703 but the possibility of altered transcription level in other tissues such as adrenal gland remains to be considered. The absence of mutations in these patients could be due to as yet undetected mutations in 5' or 3' regulatory elements of the *DAX-1* gene or due to the existence of another locus.

From previous cases, it was not clear whether HHG is a late onset manifestation of AHC which is only diagnosed at puberty by a failure of sexual maturation or a separate disorder from AHC. This study shows that eight patients older than 14 years from six families, with point mutations in *DAX-1* and without any other rearrangement detectable in the critical region, suffered from AHC and HHG. Bilateral or unilateral cryptorchidism and low serum testosterone levels (Table 2) were found in four patients below 14 years of age and can be considered as signs heralding HHG¹⁶. Therefore, we propose that mutations in *DAX-1* are also responsible for the HHG phenotype. This provides strong evidence that *DAX-1* is essential for the development of a functioning hypothalamus-pituitary-gonadal axis. The expression of *DAX-1* has not yet been tested in pituitary and hypothalamic tissue. Its known expression in testis¹⁰ could explain the HHG phenotype if the *DAX-1* putative protein controls, directly or indirectly, the hypothalamus or pituitary gland by a feedback mechanism.

TABLE 1 Deletions detected in AHC patients

Patients	Phenotype	Age of the onset of the disease	Ref.	Probes							
				4965	QST59	36	DAX-1	JK2	1A-1	22-9	GKD
V.A.	AHC	3.5 years		+	+	+	0	0	n.d.	n.d.	+
A.M.	AHC		25	0	0	0	0	+	+	+	+
G.J.	AHC		7	+	n.d.	n.d.	0	n.d.	0	+	+
J.B.	AHC		26	n.d.	0	0	0	0	n.d.	n.d.	+
3969 and 4445	AHC	4 weeks		n.d.	+	n.d.	J.F.*	+	+	+	+
3383 and 3384	GKD-AHC	neonatal		n.d.	0	n.d.	0	0	n.d.	0	0
M.C.	GKD-AHC	neonatal		+	+	+	0	0	+	+	0
B.E.N.	GKD-AHC	neonatal	27	0	0	0	0	0	0	+	0
A.S.	GKD-AHC		7	+	0	0	0	0	0	0	0
C.C. & J.H.	GKD-AHC		7	0	0	0	0	0	0	0	0
P.J.	AHC, GKD, DMD	7 days		0	0	0	0	0	0	0	0
A.B.	AHC, GKD, DMD	neonatal	28	0	0	0	0	0	0	0	0
993 and 994	GKD-AHC-MR	neonatal		n.d.	0	n.d.	0	0	n.d.	0	0
J.M.	AHC, GKD, DMD		29	0	0	0	0	0	0	0	0

* J.F.: Junction Fragment detected by *DAX-1* cDNA probe hybridization to pulsed field gel electrophoresis fragments.

Where available, patient literature references are cited (Ref.). For probe results, + and 0 indicate presence and deletion in patient DNA, respectively; n.d., not determined; MR, mental retardation. Probes 4965-5, *DAX-1* (cDNA 1.10 covering the major part of the coding and 3' untranslated sequence), 1A-1, 22-9 and GKD were hybridized to *HindIII*- or *EcoRI*-digested DNA isolated from patients using conditions previously reported^{7,24}. Probes QST59 and JK2 were tested by PCR using the primers previously described⁸. Probe 36 was tested by PCR using the following primers: 5'-ggactgtgatcatatgaagatcatcc-3' and 5'-cctttgtttacctaagcctgctctc-3'.

DAX-1 encodes a putative 470-amino-acid protein¹⁰. It is considered as a new member of the nuclear hormone receptor superfamily on the basis of a roughly 50% continuous similarity between its C-terminal portion and the ligand-binding domain of the nuclear hormone receptors. In the 258 N-terminal amino acids, a domain of 67 amino acids repeated 3.5 times has a potential DNA-binding capacity and may represent a new DNA-binding motif¹⁰.

Further investigations should elucidate the function of *DAX-1*, in particular its capacity to bind hormones and form heterodimers with other receptors *in vivo*. On this point, the missense mutation and the deletion of an amino acid in the LBD observed in two AHC-HHG patients are strong evidence for the functional relevance of this domain.

Recently, the disruption of the mouse *Ftz-F1* autosomal gene, which encodes the nuclear hormone receptor SF-1, has been shown to prevent the development of adrenal glands and gonads

in *Ftz-F1* null animals¹⁷. In the human male, disruption of *DAX-1* does not prevent the initial stages of the gonadal development but the adrenal does not differentiate beyond the fetal stage. This suggests that *DAX-1* plays a role later in development than *SF-1*. It will be interesting to analyse the possible interactions between *DAX-1* and *SF-1* during mouse development by comparing wild-type animals, *Ftz-F1* null animals and *DAX-1* null animals.

In this study, we confirm that *DAX-1* is responsible for X-linked AHC and HHG and we report different point mutations. The small size and the two-exon structure of this gene simplify the search for mutations by direct sequence analysis. AHC is a severe but treatable disease and misdiagnosis may result in death. A definitive and prompt diagnosis will now be possible for the majority of cases. AHC is genetically heterogeneous and the human homologue of the *Ftz-F1* gene could be considered as a candidate gene. In this context, the five patients presented in this

TABLE 2 Clinical details and mutation analysis of 17 families with AHC

Family	Patient	Familial case	Date of birth	Age of first diagnosis	HHG diagnosis	Mutation results (first stop codon)
1	M.A.	Yes 3 maternal uncles died between 0 and 7 years	1982	5.4 years old	Left cryptorchidism + low testosterone level	Frameshift (del-ins) and first stop codon at amino acid 401 suppression of a <i>PvuII</i> site
2	M.T.	Yes	1982	birth	n.d.	
	C.B.	Yes M.T.'s uncle	1955	10 days	HHG	Q283X suppression of a <i>PvuII</i> site
3	A.O.	Yes His cousin (L.S.) has AHC	1981	17 days	bilateral cryptorchidism	W369X
	L.S.	Yes His cousin (A.O.) has AHC	1982	15 days	No detection of testosterone, FSH, LH	suppression of a <i>HinfI</i> and a <i>PleI</i> sites
4	L.B.	Yes L.M.'s brother	1987	3.5 years	bilateral cryptorchidism + low testosterone	frameshift (549insT) and first stop codon at amino acid 184
	L.M.	Yes L.B.'s brother	1990	2.5 years	<14 yr	creation of a <i>DraI</i> site
5	B.R.	Yes	1993	8 days	<14 yr	L263X creation of a <i>BstUI</i> site
6	2115	Yes 1 maternal uncle died at age of 17 due to renal failure	1983	5 days	<14 yr	dV269
7	2687	Yes 2688's brother	1975	neonatal period	HHG	R267P† suppression of a <i>CfoI</i> site
	2688	Yes 2687's brother	1973	neonatal period	HHG	
8	2065	No	1972	3 weeks	HHG*	W235X creation of a <i>MaeI</i> site
9	2791	Yes 2792's brother	1981	neonatal period	<14 yr	frameshift (784delAA) and first codon at amino acid 204
	2792	Yes 2791's brother	1983	neonatal period	<14 yr	
10	3743	Yes 3744's uncle	1972	5 years	HHG	W172X
	3744	Yes 3743's nephew	1993	6 weeks	<14 yr	
11	3741	Yes 3742's brother	1978	10 years	HHG	frameshift (796insCAGG) and first codon at amino acid 204
	3742	Yes 3741's brother	1991	3 weeks	<14 yr	
12	2094	Yes 2095's brother	1964	3 years	HHG*	frameshift (507CG-T) and first codon at amino acid 252
	2095	Yes 2094's brother	1970	3 days	HHG*	
13	2461	No	1980	12 years	HHG	No mutation detected
14	B.V.	No	1973	5 years	HHG	No mutation detected
15	B.A.	No	1982		n.d.	No mutation detected
16	Q.N.	No	1982	9 years	Low level testosterone	No mutation detected
17	2703	Yes 4991's brother	1977	6 years	No	No mutation detected
	4991	Yes 2703's brother	1971	5 years	n.d.	

* Azoospermia.

† A transition from guanine to adenosine is present at nucleotide 1,034 which is predicted to generate the R267P missense mutation. This mutation has not been found in 40 female DNA samples used as controls.

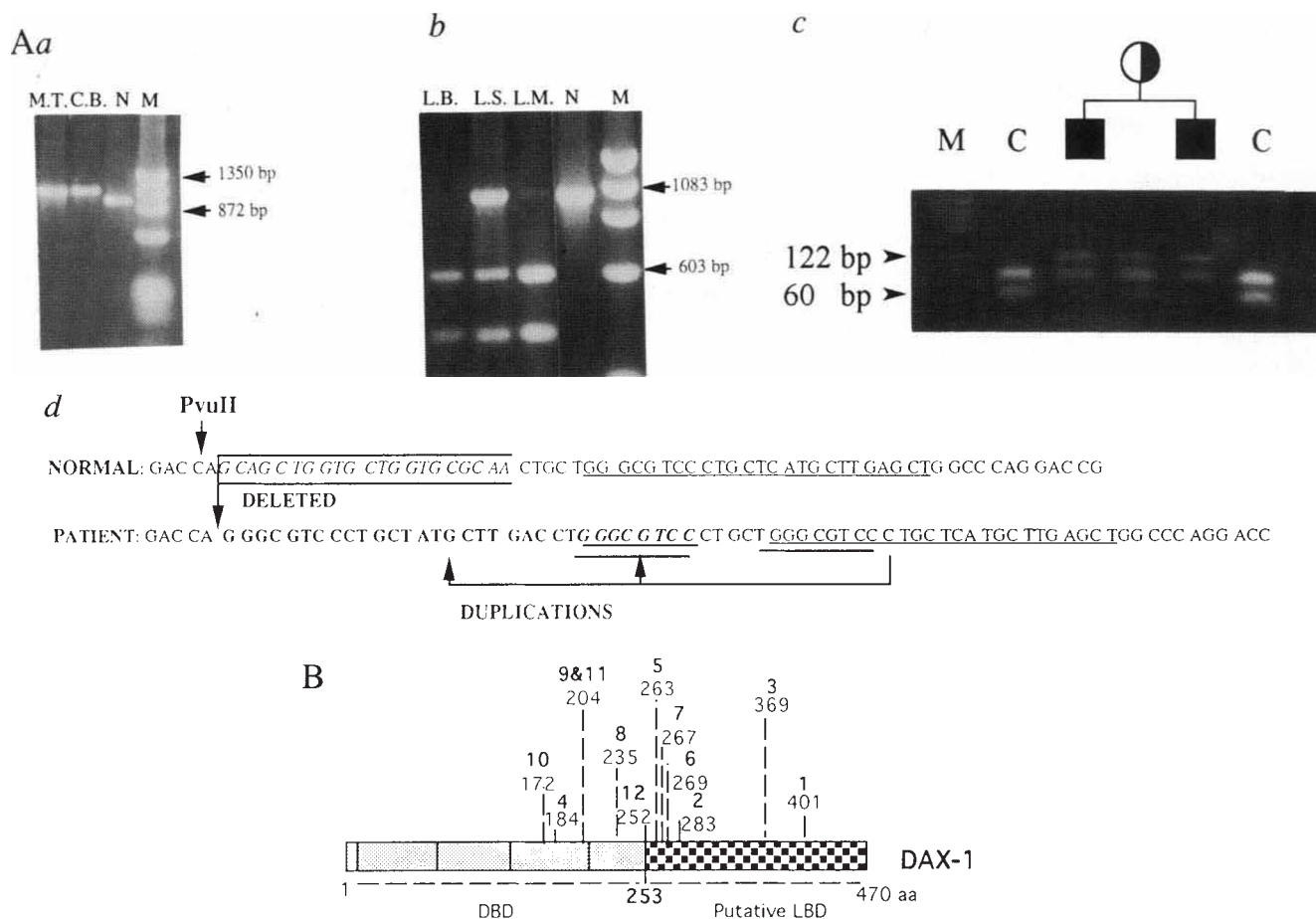


FIG. 2 Identification of point mutations in *DAX-1*. **A**, Restriction enzyme digestion patterns modified by the mutations, described in Table 2. **a**, Patients M.T. and C.B. (suppression of a *PvuII* site); **b**, patients L.B., L.M. and their mother L.S. (creation of a *DraI* site); **c**, patients 2687, 2688 and their mother (suppression of a *CfoI* site). Ethidium bromide staining of 1.5–2% agarose gel. **C**, Normal individual; **M**, ϕ X174 *HaeIII*. **d**, Sequence of the insertion-deletion revealed in patient M.A. **B**, Schematic organization of the predicted *DAX-1* protein and localization of the premature stop codons, missense or deletion codons found in patients with point mutations.

METHODS. The direct sequence analysis of PCR products excluded the possibility of sequencing artefacts resulting from nucleotide misincorporation by Taq polymerase during amplification^{11,23}. The following primer pairs were used for DNA amplification: (1), 5'-cactgggcagaa-

ctgggctac-3' and 5'-cggagcccagcatgctgcag-3'; (2), 5'-ccgcttcagttcagactgtg-3' and 5'-cgcccctagataggcactgcc-3'; (3), 5'-gctagcaaggactctgtgtg-3' and 5'-ccctcatggtgaactgcactac-3'. After a first step of denaturation (3 min, 94 °C) 35 cycles with 45 s at 94 °C, 40 s at 65 °C (2 and 3) or 68 °C (1) and 1 min 72 °C were done followed by 5 min final extension at 72 °C. Single strands were prepared using Dynabeads M 280 (Dyna) as specified by the manufacturer and directly sequenced using a T7 sequencing kit (Pharmacia). For sequencing of PCR product 'a', the following additional primers were used: (1), 5'-cgaagg-ggcccagggtgac-3'; (2), 5'-gccagggggtaagaggcgc-3'; (3), 5'-ggcagcctcag-cgggcctg-3'. To confirm the sequence, both strands were sequenced using two independent PCR reactions from patients and controls. Digestions with restriction enzymes were also done on independent PCR reactions to reveal sites created or destroyed by the mutations.

study with no mutations in *DAX-1* will be tested for mutations in the human *Fzr-FI* gene. □

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Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer in *Xenopus*

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LIKE all known LIM class homeobox genes, *Xlim-1* encodes a protein with two tandemly repeated cysteine-rich LIM domains upstream of the homeodomain¹. In *Xenopus laevis*, *Xlim-1* is specifically expressed in the Spemann organizer, whose major functions include neural induction and dorsalization of ventral mesoderm²⁻⁴. From RNA injection experiments we conclude here that: (1) the LIM domains behave as negative regulatory domains; (2) LIM domain mutants of *Xlim-1* elicited neural differentiation in animal explants; (3) mutant, and to a lesser extent wild-type, *Xlim-1* enhanced muscle formation after coinjection with *Xbra*; (4) both of these activities are mediated by extracellular signals as seen in combined explant experiments; (5) *Xlim-1* mutants activated *goosecoid* (*gsc*) expression in animal explants, but not expression of noggin or follistatin; (6) mutant *Xlim-1* elicited formation of partial secondary axes, and cooperated with *gsc* in notochord formation. Thus *Xlim-1* has latent activities, implicating it in organizer functions.

Wild-type and mutant *Xlim-1* messenger RNAs (Fig. 1a) were injected into two-cell embryos, animal explants were dissected, cultured, and the expression of neural (NCAM, *en-2*), muscle (α -actin), and cement gland (XCG7) markers was assayed; the cement gland is an anterior ectodermal derivative. The double point mutant *3m* in which both LIM domains are mutated and the LIM deletion mutant ΔNA initiated high-level expression of the neural and cement gland markers, whereas single-point mutants (*1m* and *2m*) were less effective, and wild-type was inactive (Fig. 1b). NCAM and cement gland induction was also seen histologically (see Fig. 2). In contrast, *gsc*, a homeobox gene expressed specifically in the organizer⁵, had no such activities in animal explants (Fig. 1b).

Although *Xlim-1* mutants did not induce muscle in ectodermal cells (Fig. 1b), we reasoned that it may do so in *Xbra*-expressing cells that have ventral mesodermal character and express a moderate level of α -actin⁶. The level of α -actin mRNA in *Xbra*-expressing caps was enhanced substantially by the mutants *3m* and ΔNA , and also by wild-type *Xlim-1* (Fig. 1c); the activity of wild-type *Xlim-1* is relatively weak but reproducible and concentration dependent (not shown). In contrast, *gsc* reduced rather than enhanced *Xbra*-mediated α -actin expression (Fig. 1d). These results suggest that *Xlim-1* has latent activity to initiate neural and muscle differentiation, and lead us to propose a negative regulatory function for the LIM domains, as further discussed below.

Xlim-1 is not expressed in somites and only in parts of the nervous system⁷, suggesting that its neural- and muscle-inducing effects involve intercellular signalling. Combined explant experiments tested this possibility, where one explant was lineage labelled and apposed to an explant into which different RNAs

had been injected. Although control (globin) RNA-injected recombinates did not express the neural marker NCAM, recombinates between fluorescein isothiocyanate (FITC)-labelled explants and explants carrying the *Xlim-1* mutant *3m* or *3m* + *Xbra* RNA showed substantial levels of NCAM expression both in unlabelled and, notably, in FITC-labelled cells (Fig.

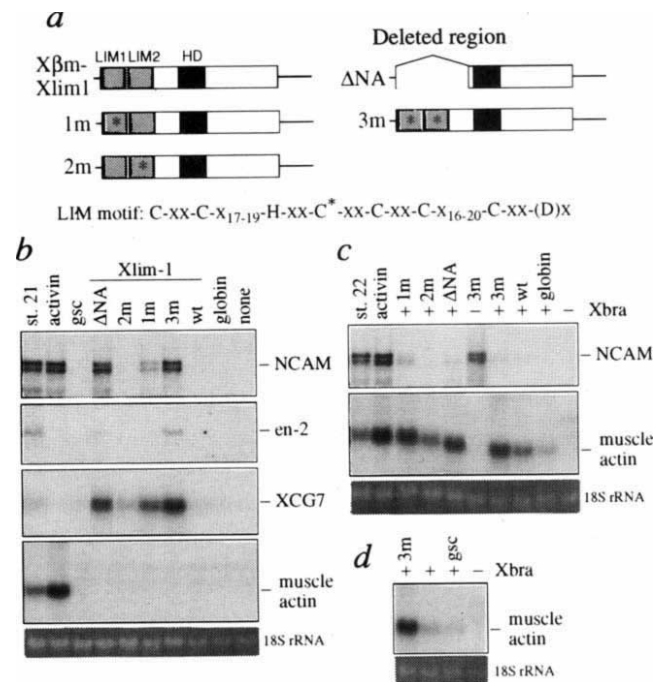


FIG. 1 Expression of neural, cement gland and muscle marker RNAs in animal explants injected with *Xlim-1* and its mutant RNAs. **a**, Mutant constructs of *Xlim-1*. Line, untranslated regions of *Xenopus* β -globin (*Xβm*); box, *Xlim-1* coding region; HD, homeodomain; asterisks, point mutation in the LIM motif changing cysteine* to glycine. **b–d**, Blots of RNAs from animal explants. RNAs from whole embryos at the indicated stages¹⁶, from uninjected explants (none), and from explants treated with 50 pM activin A were included as controls. *Xlim-1* wild type (wt) or constructs (see **a**), globin and *gsc* RNAs were injected as indicated; coinjection of *Xbra* RNA is indicated by a plus sign. Each RNA was injected at 1 ng per embryo. The blots were sequentially hybridized with NCAM, *en-2*, XCG7 and muscle actin probes. NCAM probe detects several RNAs, the largest two bands (9.5 and 7.0 kb) being shown; only the 3.0 kb mRNA of *en-2* is shown. The ethidium bromide-stained 18S ribosomal RNA for each gel is shown as a loading control.

METHODS. A DNA fragment containing the *Xlim-1*¹ coding sequence was generated by polymerase chain reaction (PCR) using primers with a *Nco*I site at the initiation codon and *Hind*III–*Eco*RI site after the termination codon. The *Nco*I–blunt-end fragment of the PCR product was cloned into *Nco*I–*Bst*EII-digested *Xβm* vector¹⁷ to construct *Xβm-Xlim1*. Point mutations were introduced with mutated oligodeoxynucleotides and an *in vitro* site-directed mutagenesis kit (Clontech). To make the deletion construct ΔNA , *Nco*I and *Asel* sites in *Xβm-Xlim1* were used. All constructs were verified by sequencing. When we refer to *Xlim-1* or wild-type (wt) *Xlim-1* we mean *Xβm-Xlim1*. Other plasmids used were *pgsc*⁵, *pSP64-Xbra*⁶ and *Xβm*¹⁷. Capped RNAs were synthesized using linearized plasmid DNAs as templates, and the Megascript (Ambion) *in vitro* transcription kit with m⁷-GpppG. The amount of RNA was determined by ethidium bromide staining in comparison with standard RNA. For injection, RNA was dissolved in 10 mM HEPES (pH 6.8), 88 mM NaCl. The RNA solution (5 nl) was injected into both blastomeres at the 2-cell stage close to the animal pole. At the blastula stage (about stage 8.5¹⁶), 8 to 12 animal explants per group were dissected and cultured¹ until sibling embryos reached tailbud stages 21–23. RNA extraction and blotting were as described¹, with 5 μ g RNA per lane. Hybridization probes were *Xenopus* NCAM cDNA N1¹⁸, *Xenopus* 1.4 kb *en-2* cDNA¹⁹, XCG7 cDNA²⁰ and muscle actin cDNA²¹; washed filters were analysed by a PhosphorImager (Molecular Dynamics).

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2a). This indicates that *3m*-RNA-injected cells can induce neural differentiation in adjacent cells. Cement gland (thin arrow, Fig. 2a) was also induced in FITC-labelled caps in combination with *3m*-RNA-injected caps (13 of 18), but not in control recombinates (0 of 16). Although caps injected with both *3m* and *Xbra* did not express NCAM, they displayed strong NCAM-inducing activity towards adjoining FITC-labelled caps (Fig. 2a).

Explants expressing *3m* could induce muscle differentiation in adjoining *Xbra*-expressing caps (Fig. 2b). Only 2 of 18 FITC-*Xbra* caps combined with control (globin) caps stained for muscle in a small region; one of these positive caps is shown (Fig. 2b). In contrast, intense staining confined to the *Xbra*-expressing cells was seen in 11 of 18 combinations of FITC-*Xbra* and *3m*-expressing explants.

Pattern formation and tissue differentiation during gastrulation are mediated through the activity of rapidly induced regulatory genes⁴. *Xlim-1* mutants could activate *gsc* but none of the other genes tested, including *Xlim-1* itself (Fig. 3a); in particular, the neural and dorsalizing inducer noggin⁸ and the neural inducer follistatin⁹ were not induced. Thus, the dorsalizing and neuralizing signals generated by *3m*-expressing cells are likely to be transmitted by molecules other than noggin or follistatin.

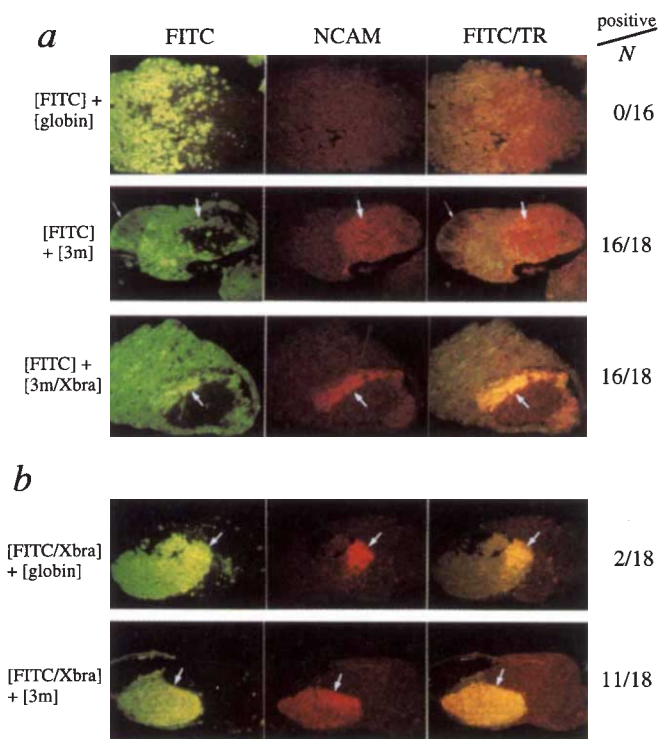


FIG. 2 Neural and muscle induction in animal cap recombinates. a, Neural and b, muscle differentiation as seen by immunocytochemistry in sections of recombinates at equivalent stage 26–28 (tailbud). Combinations are indicated at the left of the row where the materials injected into each embryo are enclosed in brackets, and the two explants in the recombinant are separated by a plus sign. The filter used for visualization of fluorescence is shown above each column. Green colour derived from FITC, and red from Texas red (TR) from anti-body staining, using anti-NCAM antibody 4d²² or muscle-specific antibody 12/101²³. The third column used a dual-band filter that shows areas positive for both dyes as yellow–orange. Arrows indicate double-stained regions, that is, NCAM or 12/101 expression in the FITC-labelled halves. Thin arrows in a indicate cement gland. Incidence of staining for NCAM or 12/101 in FITC-positive cells is shown at the right; N, total number of combined caps examined in three independent experiments. Additional controls not shown are as follows: NCAM was not induced in the [FITC] + [Xbra] combination (0 of 16); in the combination [FITC/Xbra] + [Xbra], muscle staining was seen in 1 of 17 cases. Methods used were as described previously²⁴.

Whereas *gsc* is activated by *Xlim-1* mutants, *gsc* does not activate *Xlim-1* in animal caps (Fig. 3b), but does so in the ventral marginal zone (VMZ; Fig. 3c). Presumably the VMZ contains cofactors that cooperate with *gsc* in gene activation.

Because the *Xlim-1* mutant *3m* has activities leading to induction of neural tissue, cement gland and muscle in animal explants, we examined whether it could elicit these functions in whole embryos. Injection of *3m* RNA into the ventral equatorial region gave rise to secondary axes which contained somites, neural tissue, and cement gland but rarely notochords; these results were similar to those obtained by *gsc* RNA injection¹⁰ (Fig. 4a). Strong synergism was observed between *gsc* and *3m* in notochord formation so that almost all embryos injected with a combination of both RNAs formed at least one and often two ectopic notochords (Fig. 4a, b).

It has been reported that the LIM domains inhibit the sequence-specific DNA-binding activity of the homeodomain in Mcc-3 and Isl-1 in *in vitro* and cotransfection assays^{11,12}. These reports are consistent with our functional data in which wild-type *Xlim-1* had weak muscle-inducing and no neural-inducing activity whereas LIM domain point or deletion mutants were highly effective. If wild-type *Xlim-1* is a largely inactive form, how is it activated *in vivo*? A clue may come from the strong activation of the insulin gene by the LIM-homeodomain protein Lmx-1 in combination with the helix-loop-helix protein Pan1/E47¹³. Likewise, *Xlim-1* may require one or several protein coactivators.

In mRNA injection experiments we observed the following five activities of the *Xlim-1* mutant *3m* (see Fig. 1a): (1) neural induction, (2) cement gland formation, (3) *gsc* gene activation, (4) muscle induction in *Xbra*-expressing cells, and (5) notochord formation in combination with *gsc*. The above activities are all known as attributes of the Spemann organizer. Similar activities

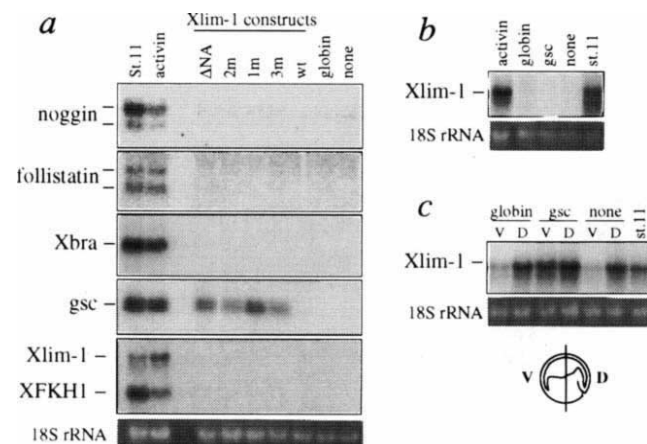


FIG. 3 Activation of early response genes by *Xlim-1* constructs at the gastrula stage. a, Expression of noggin, follistatin, *Xbra*, *gsc*, *Xlim-1* and *XFKH1* in animal explants injected with *Xlim-1* construct RNAs at 0.7 ng per embryo. The RNA blot included positive controls (whole embryo, and 50 pM activin A-treated explants), and negative controls (globin and uninjected explants). b, Absence of expression of *Xlim-1* in animal explants injected with *gsc* RNA at 1 ng per embryo. c, Expression of *Xlim-1* in whole embryos injected with *gsc*. Embryos were injected with *gsc* or *globin* RNAs at 1 ng per embryo into the ventral equatorial region at the 4-cell stage and dissected at the gastrula stage (st. 11) into ventral (V) and dorsal (D) halves. The blot included uninjected control (none).

METHODS. The 1.3 kb 3'UTR of *Xlim-1* cDNA pXH32-3¹ was generated by PCR with specific primers (5'-TAGCCTAAGCTGGACAA, forward; 5'-AA-ATATCAGAATTTTCTATTT, reverse); this region did not hybridize with the injected *Xβm-Xlim1* RNA. The other probes were isolated from full-length noggin cDNA A3²⁵, Follistatin cDNA XSF-319⁹, *pgsc*⁵, *XFKH1* cDNA²⁶. Full-length *Xlim-1* cDNA pXH32-3 was used for blots b, c.

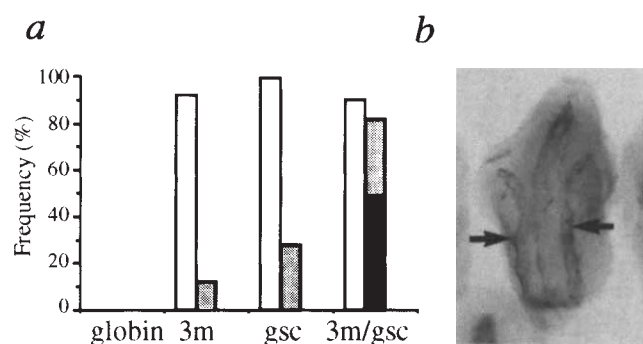


FIG. 4 Secondary axis formation. *a*, Incidence of ectopic somite and notochord formation in embryos injected with *Xlim-1* mutant 3m, *gsc*, or both. Open bars, ectopic somite; grey bars, one ectopic notochord; black bar, two ectopic notochords. The 3m-induced secondary axes also contained a high incidence of neural tissue and cement gland (not shown). The total number examined was 28 (globin), 100 (3m), 72 (*gsc*) and 39 (3m/*gsc*). *b*, Example of two ectopic notochords formed in an embryo coinjected with 3m and *gsc* RNAs, as visualized by whole-mount staining with the notochord-specific antibody Tor70²⁷ at stage 27/28. Dorsal view, anterior is up; arrows indicate ectopic notochords. METHODS. Embryos were injected with RNA into two blastomeres at the 4-cell stage in the ventral equatorial region. The amount of RNA was 0.5–1.75 (globin), 0.5–1.0 (3m) and 0.38–0.75 (*gsc*) ng per embryo. In coinjection of 3m and *gsc*, the ratio of 3m to *gsc* was 1 to 0.75. At tailbud stages, embryos were analysed by whole-mount immunostaining²⁸ after bleaching with 10% H₂O₂. In some injected embryos, blastopore closure was incomplete, probably due to gastrulation arrest; such embryos were discarded.

are displayed by *gsc* when its mRNA is injected into the ventral equatorial region^{3,14}, but not in animal ectodermal cells (ref. 15 and our results). Cells expressing 3m send inducing signals to adjacent cells (Fig. 2); therefore, it is likely that LIM domain mutants of *Xlim-1* activate genes encoding neural- and muscle-inducing factors. These putative molecules appear to be distinct from noggin or follistatin (Fig. 3a), implicating new signalling factors in organizer function. □

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Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by *Wnt-4*

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THE kidney has been widely exploited as a model system for the study of tissue inductions regulating vertebrate organogenesis^{1,2}. Kidney development is initiated by the ingrowth of the Wolfian duct-derived ureteric bud into the presumptive kidney mesenchyme. In response to a signal from the ureter, mesenchymal cells condense, aggregate into pretubular clusters and undergo an epithelial conversion generating a simple tubule. This then undergoes morphogenesis and is transformed into the excretory system of the kidney, the nephron. We report here that the expression of *Wnt-4*, which encodes a secreted glycoprotein, correlates with, and is required for, kidney tubulogenesis. Mice lacking *Wnt-4* activity fail to form pretubular cell aggregates; however, other aspects of mesenchymal and ureteric development are unaffected. Thus, *Wnt-4* appears to act as an autoinducer of the mesenchyme to epithelial transition that underlies nephron development.

Wnt-signalling is thought to play diverse roles, regulating pattern, cell fate choices and mitotic activity in different embryonic tissues. To address the possibility that Wnt-family members may participate in development of the vertebrate kidney, we examined expression of 12 mouse Wnt genes by *in situ* hybridization (data not shown). One of these, *Wnt-4*, was expressed in the kidney mesenchyme and its derivatives. *Wnt-4* messenger RNA was detected in condensed mesenchymal cells on both sides of the stalk of the ureter at 11.5 days postcoitum (d.p.c.) and correlated to the site where the first pretubular cell aggregates form² (Fig. 1a–c). During subsequent development, this expression pattern was repeated in newly forming aggregates, as well as their simple epithelial tubular derivatives (Fig. 1d–f).

Wnt-4 expression was also detected in comma-shaped bodies, but later became restricted in descendent S-shaped bodies to the region where epithelial fusion was occurring with the collecting duct, and was lost after fusion was completed (Fig. 1e, f and data not shown). At later stages, *Wnt-4* expression became confined to the periphery of the kidney where new tubules were forming (Fig. 1f). Interestingly, in both the chick (Fig. 1g) and mouse³ (data not shown), the dorsal spinal cord, a potent inducer of kidney tubule differentiation^{1,2}, also expressed *Wnt-4*.

Development of the metanephric kidney is preceded by the formation of a mesonephric kidney. In the mouse, this is a vestigial structure, whereas in avian species, it is an elaborate functional organ during embryonic life. *Wnt-4* was expressed throughout mesonephric tubulogenesis from the aggregating mesenchyme until fusion of the epithelial tubules with the Wolfian (mesonephric) duct (Fig. 1g, h). Thus, it is likely that the role of *Wnt-4* is conserved in the development of the mesonephric and metanephric kidneys.

To examine *Wnt-4* function, we used gene targeting in mouse embryonic stem (ES) cells to replace the third coding exon of the *Wnt-4* gene with a selection cassette containing neomycin phosphotransferase (neo) (Fig. 2), thereby generating a likely

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null allele. Adult mice heterozygous for this disrupted allele showed no obvious phenotype. However, homozygous pups died within 24 h of birth. As *Wnt-4*^{-/-} embryos were recovered at the expected mendelian frequency at late stages of gestation (data not shown), *Wnt-4* is not essential for *in utero* development. All neonatal and 18.5 d.p.c. *Wnt-4*^{-/-} embryos contained small agenic kidneys (compare with adjoining adrenal gland in Fig. 3a, b), consisting of undifferentiated mesenchyme interspersed with branches of collecting duct epithelium (Fig. 3c-f). Thus, death of homozygous pups is almost certainly due to the lack of kidney function.

To address the role of *Wnt-4*, we analysed kidney development in mutant embryos. As expected, initial development of the primary condensate around the tips of the ureteric bud appeared histologically normal (Fig. 3g, h), suggesting that induction of kidney mesenchyme by the ureter was unaffected. Early on the 15th day of pregnancy, no gross difference was observed in the size of kidneys between *Wnt-4*^{+/-} and *Wnt-4*^{-/-} embryos (Fig. 3i-l). Like their normal litter mates, mutant embryos had undergone considerable branching of the ureteric epithelium. However, the kidney mesenchyme remained morphologically undifferentiated, lacking pretubular aggregates and more developed tubules (Fig. 3j, l). Later on the same day, *Wnt-4*^{-/-} kidneys were clearly growth retarded (Fig. 3m, n) and the mesenchyme persisted in a morphologically undifferentiated state (data not shown). Three out of ten *Wnt-4*^{-/-} embryos

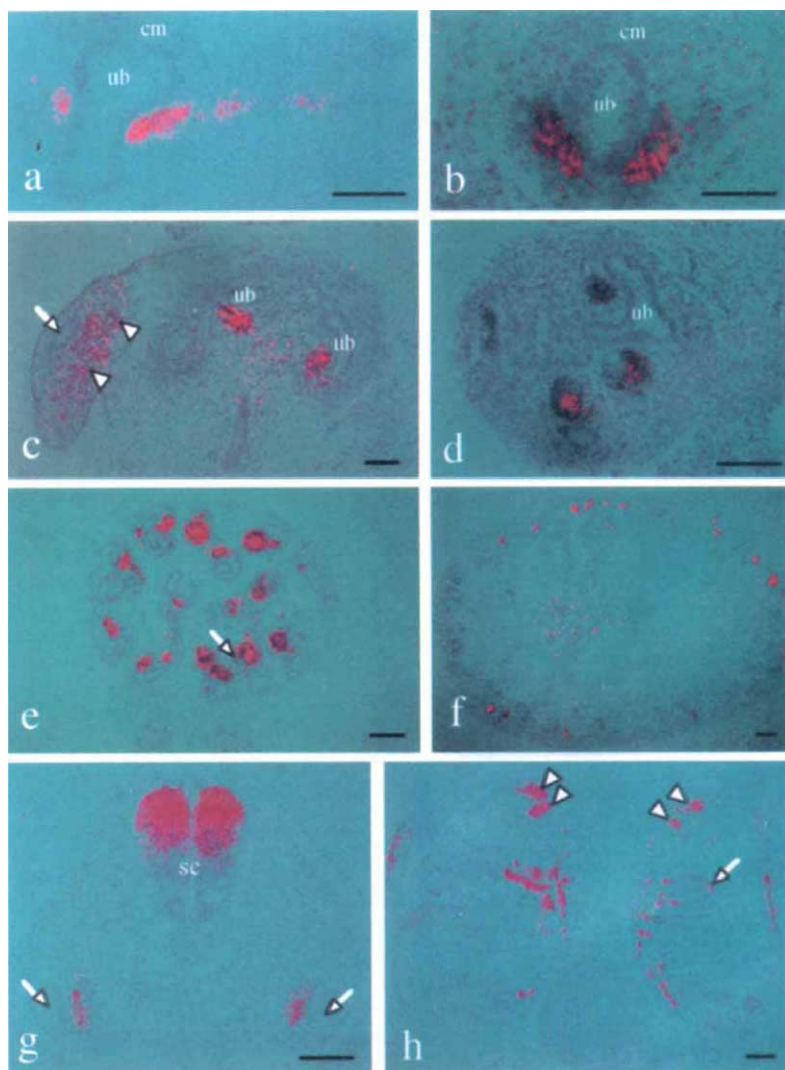
examined on the 15th day of pregnancy showed no histological evidence of mesenchymal aggregation. In the remainder, a few poorly developed aggregates were observed (less than 10% of the number of aggregates scored in *Wnt-4*^{+/-} or *Wnt-4*^{-/-} litter mates). We never observed comma-shaped or S-shaped bodies.

Kidney development was further investigated by the analysis of a variety of molecular markers. Presumptive and induced kidney mesenchyme, as well as differentiating glomerulae, express the *Wilms tumor (WT-1)* before mesenchymal condensation^{4,5}. Initial expression of *WT-1* was unchanged in *Wnt-4*^{-/-} embryos (Fig. 4a, b), persisting until at least 14.5 d.p.c. However, throughout this period, expression was only observed in the mesenchyme (Fig. 4c, d). *N-Myc*, an early marker of tubule induction, is first detected in a subpopulation of condensed mesenchyme and is then upregulated in epithelial structures which presumably arise from these cells⁶. *Pax2* has also been suggested to regulate induction and epithelial conversion of the kidney mesenchyme^{7,8}. *N-Myc* and *Pax2* were expressed until at least 14.5 d.p.c. in *Wnt-4*^{-/-} embryos; however, no expression was detected in tubules (Fig. 4e-l). These data suggest that the mesenchymal cells fated to form tubules were induced but failed to convert into epithelial structures.

Failure of pretubular aggregation is also supported by analysis of *Wnt-4* and *Pax-8* expression. Although *Wnt-4* expression appears to precede that of *Pax8* (data not shown) both genes share an identical distribution in the pretubular aggregate and

FIG. 1 Expression of *Wnt-4* during morphogenesis of the metanephric kidney of the mouse and mesonephric kidney of the chick. *In situ* hybridization with ³⁵S mRNA probes was used to detect mouse (a-f) and chick (g, h) *Wnt-4* mRNA. a, In the metanephric kidney *Wnt-4* expression is first seen at 11.5 d.p.c., in condensed mesenchymal (cm) cells on both sides of the invading ureteric bud (ub), preceding the formation of the first pretubular aggregates. b, A transverse section through a similar stage kidney to that shown in a. c, *Wnt-4* expression becomes restricted to pretubular aggregates on initiation of ureteric bud branching (12.5 d.p.c.). Expression is also seen in the mesonephric mesenchyme (arrowheads), adjacent to the mesonephric duct (arrow). d, By 13.5 d.p.c., *Wnt-4* expression is apparent in primitive tubular aggregates. e, On subsequent branching of the ureter (14.5 d.p.c.), *Wnt-4* expression is induced in the assembling aggregates. Expression is also seen in comma-shaped bodies (arrow), and more advanced stages of tubular morphogenesis. f, Later during development (16.5 d.p.c.) *Wnt-4* expression is confined to the cortex of the kidney where new tubular aggregates are still forming. g, In the stage 20 chick embryo, *Wnt-4* expression is induced in the mesonephric mesenchyme of the mesonephros, adjacent to the mesonephric duct (arrow). *Wnt-4* is also expressed in a graded distribution in the dorsal half of the spinal cord (sc). h, At stage 27-29, development of the mesonephric kidney is advanced and *Wnt-4* expression is apparent in early stage aggregates (arrowheads), as well as in more advanced stages. By the time that the tubules fuse with the mesonephric duct, *Wnt-4* expression becomes restricted to the tip of the tubule at the point of fusion (arrow) and is lost after fusion. Scale bars, 10 μ m (a-c, g, h); 100 μ m (f).

METHODS. *In situ* hybridization analysis was according to published procedures²⁴ using a *Wnt-4* mRNA probe⁵. The chick *Wnt-4* cDNA consisted of a roughly 400 base pair fragment generated by polymerase chain reaction (J. McMahon and A.P.M., unpublished data).



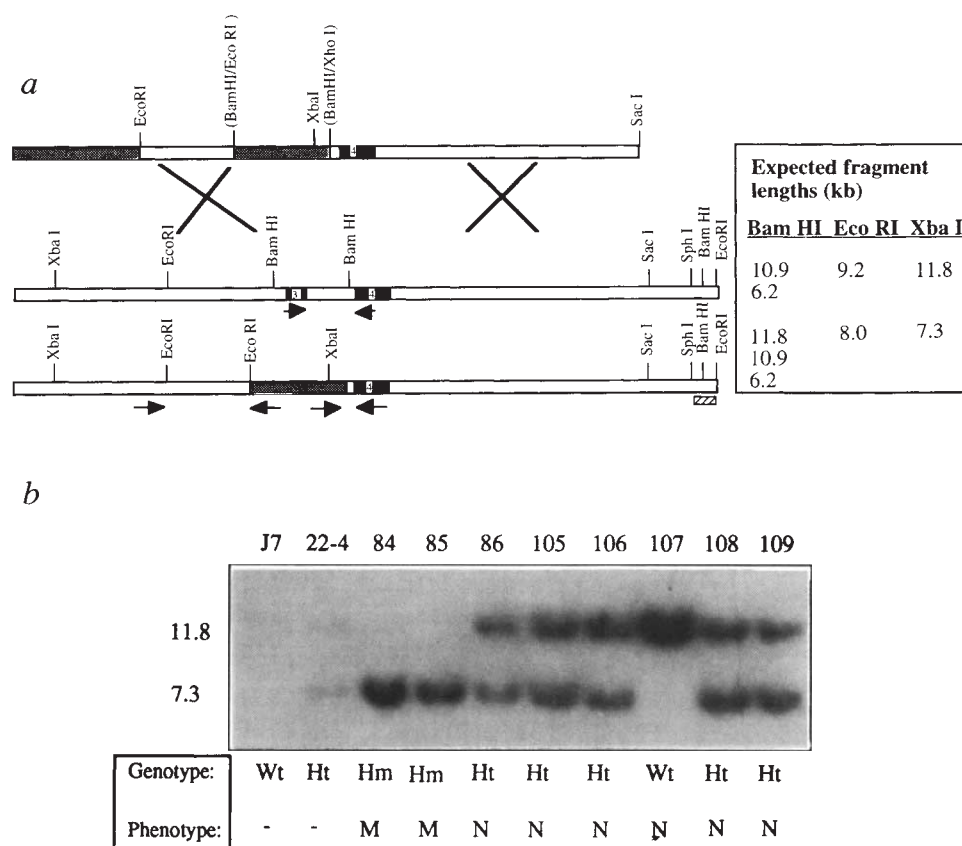
developing tubule⁹ (compare Fig. 4o and s). In mutant embryos, expression of both of these genes was never detected in kidneys scored as completely negative for tubule formation by histological analysis and was only detected in the rare case of a small and poorly organized aggregate (Fig. 4m-t). As normal *Wnt-4* expression was observed in several tissues that were not overtly affected in *Wnt-4*^{-/-} embryos (floorplate, spinal cord and mesonephric mesenchyme; Fig. 4n and data not shown), it is clear that the targeted allele was not transcribed. Thus, the failure to detect cells initiating *Wnt-4* expression suggests that *Wnt-4* may be required for full activation of its own expression in the kidney mesenchyme. Alternatively, cells lacking *Wnt-4* activity might rapidly adopt another fate. Further, *Wnt-4* is required,

directly or indirectly, for the activation and/or maintenance of *Pax8* expression. Activation of *Pax-8*, a paired domain containing transcription factor, may be critical for the regulation of other genes essential for tubule formation.

Kidney development is critically dependent on the ureter. For example, mice lacking *c-ret* activity, a receptor protein tyrosine kinase normally expressed in the ureteric bud¹⁰, die because of complete or partial failure of kidney development¹¹. Expression of *c-ret* and *c-ros*, a second receptor tyrosine kinase transcribed in the ureteric epithelium^{12,13}, was activated and maintained in the tips of the ureteric bud in mutant kidneys (Fig. 4u-y; data not shown). This result, together with the observed branching of the ureter, supports the conclusion that development of the

FIG. 2 Gene targeting at the *Wnt-4* locus. **a**, Schematic representation of the *Wnt-4* targeting vector (upper), *Wnt-4* locus (middle) and expected recombination event (lower). Successful gene replacement by the targeting vector results in the deletion of roughly 2.2 kb of *Wnt-4* genomic sequence, including all of the coding sequence derived from exon 3 (amino acids 106 to 196 in the predicted *Wnt-4* protein sequence²⁵), and insertion of a *PGK-neo* selection cassette²⁶, in the same transcriptional orientation as that of the *Wnt-4* gene. The arrows indicate PCR primers, and the hatched box a DNA probe used to identify putative targeted ES cell clones. **b**, Southern blot analysis of gene targeting at the *Wnt-4* locus and transmission of the targeted allele. An 11.8 kb *Xba*I fragment, indicative of the wild type *Wnt-4* allele, is detected in the parental CJ7 ES cell line²⁷. An additional fragment of 7.3 kb, corresponding to the targeted allele, is detected in DNA from clone 22-4. Additional Southern analysis with *Bam*HI and *Eco*RI digests, and with a 5' flanking probe, confirmed that the predicted targeting event occurred in clone 22-4 (data not shown). Lanes 84 to 109 represent selected DNA samples from 18.5 d.p.c. progeny derived from intercrosses between mice heterozygous for the targeted *Wnt-4* allele. Agenesis of the kidney was apparent in all homozygous (Hm) embryos at this stage. No phenotype was observed in heterozygous (Ht) or wild-type (Wt) littermates.

METHODS. The gene targeting construct, containing 1.6 kb of 5' and 4.5 kb of 3' homology, was transfected into the CJ7 ES cell line²⁷ and clones selected using positive (*PGK-neo* cassette²⁶) and negative (*MC1TK* cassette²⁸) selection with G418 and FIAU, respectively, as described previously²⁹. Surviving clones were screened for homologous recombination by PCR using a 5' oligonucleotide homologous to sequences in intron 2 of *Wnt-4* (5'-GCGTTCTGCCTCCCTCCTGCGGG-3'), which lies just upstream of the 5' homology region in the targeting construct, and a 3' oligonucleotide (5'-GGGAGCCGGTTGGC-GCTACCGTGG-3') homologous to *PGK* promoter sequences in the *PGK-neo* cassette (arrows in **a**). Sample preparation and PCR amplification was as described previously²⁹, except that the initial PCR was performed on pools of 5 samples. Gene replacement at the *Wnt-4* locus leads to the amplification of a 1.6 kb 5' homology region. Because a band of the expected size was not observed with ethidium bromide staining, the PCR products were transferred to nylon membranes and hybridized with a ³²P-labelled oligonucleotide homologous to sequences within the amplified region (5'-GCTGAGTGGCTAGAGCCAG-3'). One positive pool



(22) was identified; on further analysis, one sample of the five clones in this pool was PCR positive. The overall targeting frequency was ~1 in 200 clones which survived double selection. DNA was prepared from this clone (22-4) and analysed for correct targeting by Southern blot analysis using both 5' and 3' single copy probes and multiple diagnostic restriction enzyme digests. Having verified that homologous recombination had occurred as expected, 22-4 cells were used to generate chimeras and germ-line transmission of the targeted allele was monitored by Southern analysis using the indicated probe (hatched box in **a**). All subsequent genotyping was done by Southern blot or by PCR. The wild-type *Wnt-4* allele was detected by PCR using a 5' oligonucleotide (5'-CTTCACAACAACGAGGCTGGCAGG-3') and a 3' oligonucleotide (5'-CACCCGCATGTGTGCAAGATGG-3') homologous to nucleotides 655-679 in exon 3 and 683-706 in exon 4 of the *Wnt-4* cDNA²⁵. The targeted *Wnt-4* allele was identified by using a 3' primer homologous to sequences within the *PGK-neo* cassette (5'-GCATTGTCTGAGT-AGGTGTCATTC-3') and the *Wnt-4* exon 4 primer described above. PCR amplifies fragments of about 400 bp (targeted allele) and 700 bp (wild-type allele). PCR was done for 45 cycles of: 1 min at 93 °C, 65 °C for 30 s, 72 °C for 30 s, followed by 5 min at 72 °C.

ureteric epithelium, at these stages, is independent of both *Wnt-4* expression and tubule formation.

Up to the 15th day of pregnancy, there is no visible decrease in size or increase in cell death in mutant kidneys. This period is normally marked by a general decrease in cell proliferation in developing tubules². Thus, it seems unlikely that *Wnt-4* regulates cell growth or survival. Together, our evidence is most consistent with a model in which *Wnt-4* acts as an autoactivator of the mesenchymal to epithelial transition. Thus, initiation of *Wnt-4* expression in a cluster of induced cells leads to these same cells forming the pretubular aggregate. Continued expression of *Wnt-4* suggests an additional role in later stages of tubule morphogenesis which cannot be addressed at present.

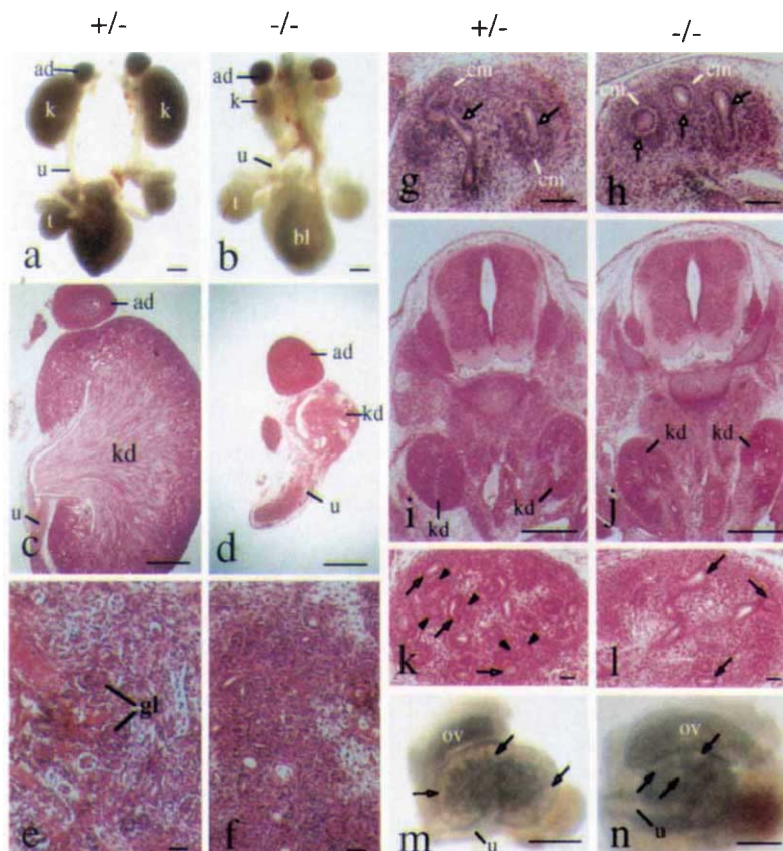
Clearly, some mechanism must account for the discrete localization of *Wnt-4* to the aggregates. One possibility is that induction of mesenchyme by the ureter leads to both the activation of *Wnt-4* expression and the competence to respond to this signal. Thus, only those cells in the distal mesenchyme that were previously in contact with the ureter are induced to aggregate by a *Wnt-4* signal. Indeed, aggregating cells are unable to elicit tubule formation in uninduced nephrogenic mesenchyme¹⁴. Alternatively, aggregating mesenchymal cells may inhibit neighbouring cells from adopting the same fates.

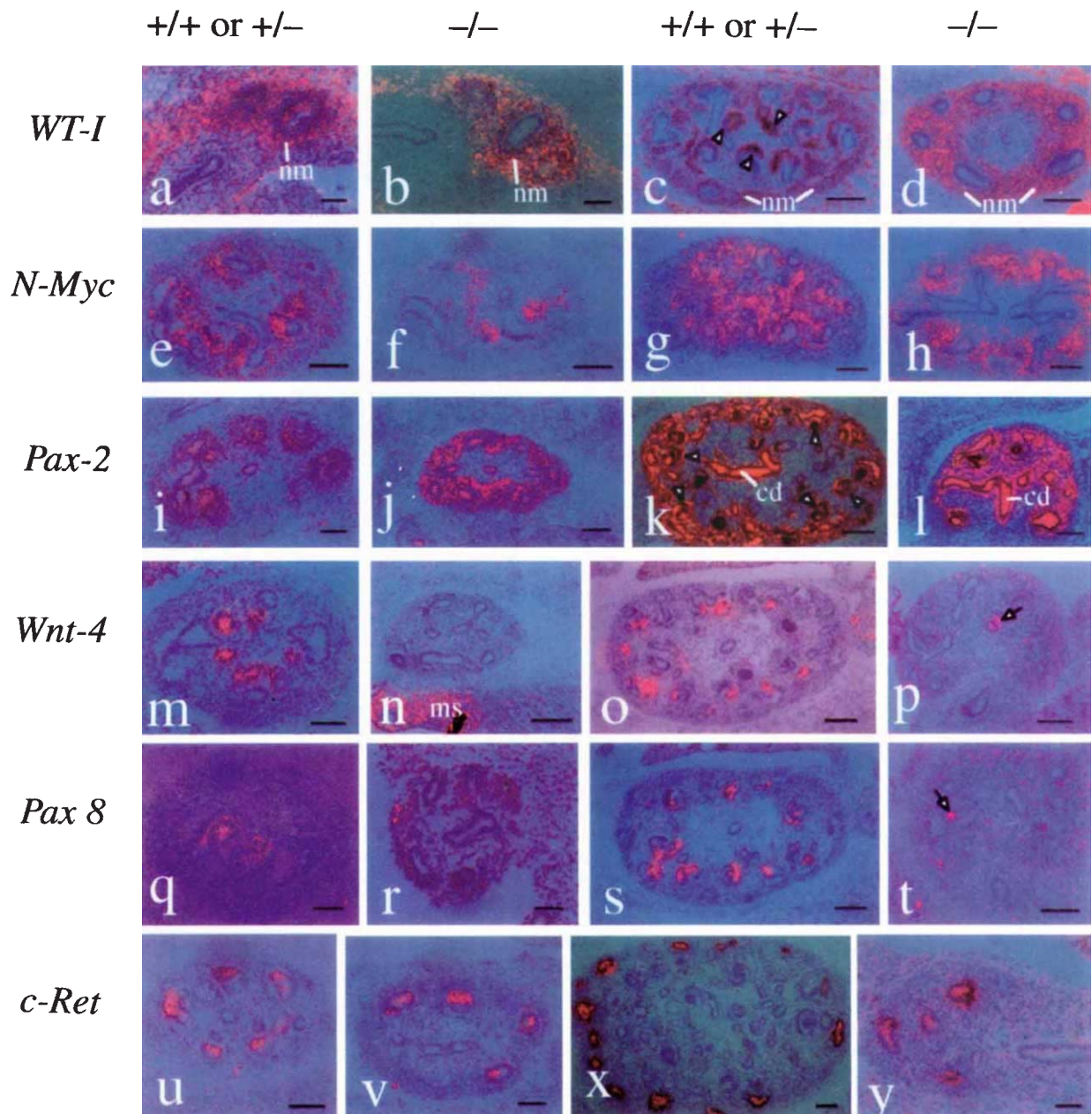
One likely step in the mesenchyme to epithelial transition is regulation of cell adhesion. Interestingly, ectopic expression of *Wnt-4* (R. Moon, personal communication) and *Wnt-5a*, in the *Xenopus* embryo¹⁵, may increase cell adhesiveness. Moreover, *armadillo*, which is a *Drosophila* homologue of beta-catenin, a cytoplasmic protein essential for E-cadherin-mediated adhesion^{16,17}, is required for *wingless* (the *Drosophila* orthologue of *Wnt-1*) signalling^{18,19}. *Wnt-1* has been shown to modulate cell adhesion *in vitro*^{20,21}, and the expression of the cell adhesion molecule, E-cadherin *in vivo*²². Interestingly, E-cadherin is expressed in the early kidney tubule, after activation of *Wnt-4* and *Pax-8* expression²³. Hence, it is tempting to speculate that

FIG. 4 Expression of mesenchymal and epithelial marker genes in kidneys of *Wnt-4* mutants. *WT-1* is expressed in the undifferentiated nephrogenic mesenchyme (nm) at 12.5 (a) and 14.5 (c) d.p.c. in *Wnt-4*^{+/+} or *+/+* embryos. Expression is also seen in early and late stages of tubule morphogenesis at 14.5 d.p.c. (arrowheads in c). *WT-1* expression in the kidneys of *Wnt-4*^{-/-} embryos is restricted to morphologically undifferentiated mesenchyme (b, d). *N-Myc* expression at 12.5 d.p.c. localizes to a subset of induced mesenchyme in kidneys from wild-type (e) and homozygous mutant embryos (f). Expression is also seen in more advanced stages of tubule development in the normal kidney (g), but not in kidneys from *Wnt-4*^{-/-} embryos (h). *Pax2* shows complex expression, first in the invading ureteric epithelium and condensed mesenchyme (i), then also in comma- and S-shaped bodies (arrowheads), and proximal and distal collecting duct (cd) epithelium, at 14.5 d.p.c. (k). Expression is unchanged in the ureter and kidney mesenchyme of *Wnt-4* mutants at 12.5 d.p.c. (j), and extends into the collecting duct epithelium at 14.5 d.p.c. (l); however, epithelial expression is limited to ureteric derivatives. *Wnt-4* (m-p) and *Pax-8* (q-t) are both expressed in pre- and early tubular aggregates (m, q), as well as in comma- and S-shaped bodies (o, s). In *Wnt-4* mutants only occasional expression of either gene is seen in the kidney and expression is restricted to poorly developed aggregates (arrowed in p and t). o and s are adjacent sections. *Wnt-4* expression in the central nervous system and mesonephric mesenchyme (ms in n) is unaltered in *Wnt-4*^{-/-} embryos. Expression of *c-ret* localizes to the tips of the ureter at 12.5 (u) and 14.5 (x) d.p.c. In *Wnt-4* mutants, expression persists at the tips of the newly branched ureter at 12.5 (v) and until at least 14.5 d.p.c. (y), suggesting that development of the ureteric bud is not dependent on *Wnt-4*. Scale bar, 10 μ m.

METHODS. *In situ* hybridization to embryo sections was as described²⁴. The probes used were as described: *WT-1*⁵, *N-myc*³⁰, *Pax-2*⁷, *Wnt-4*³, *Pax-8*⁹, *c-Ret*¹⁰.

FIG. 3 Histological analysis of kidney development in *Wnt-4* mutant embryos. Whole-mount views (a, b, m, n) and histological sections (c-l) of kidneys from embryos taken at 18.5 d.p.c. (a-f), 12.5 d.p.c. (g, h), and early (i-l) and late (m, n) on the 15th day of pregnancy. e, f, High-power views of c and d, respectively. k, l, High-power views of i and j, respectively. For description, see text. Arrows indicate the invading and branching ureteric epithelium; arrowheads, epithelial tubular aggregates. ad, Adrenal gland; bl, bladder; cm, condensed mesenchyme; gl, glomerulus; kd, kidney; ov, ovary; t, testis; u, ureter. Scale bars, e, f, g, h, k, l, 10 μ m; a-d, i, j, m, n, 100 μ m. METHODS. Embryos were collected into PBS, a sample of the yolk sac or liver was removed for genotyping and the remaining embryo, or isolated kidneys, were fixed in Bouin's fixative. Samples were dehydrated, embedded in wax and sectioned at 3 to 6 μ m. Sections were dewaxed, rehydrated and stained with haematoxylin and eosin.





Wnt-4 may regulate mesenchymal aggregation and tubule formation through modulation of cell adhesion factors. ☐

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The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest

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THE pattern of skeletal structures and muscles in the branchial region of the head is profoundly influenced by the neural crest¹, whose cells arise at discrete segmental levels of the chick hindbrain: specifically, rhombomeres (r)1 + 2, r4 and r6, whereas r3 and r5 are crest-depleted². We have demonstrated that an interaction between even-numbered rhombomeres and r3/r5 effects this depletion of neural crest, resulting in the sculpting of discrete migratory streams of neural crest³. This mechanism acts through increased expression of *msx2* and the induction of apoptosis in dorsal cells of r3 and r5 (ref. 3) (Fig. 1A). Here we demonstrate that the signalling molecule *Bmp4* is expressed in r3 and r5 and is dependent on the neighbouring rhombomeres. Addition of recombinant BMP4 protein to explant cultures of r3 or r5, which produce neural crest when isolated from their neighbouring rhombomeres, upregulates *msx2* and reinstates apoptosis in the neural crest population.

BMP4 is a member of the transforming growth factor- β (TGF- β) superfamily of signalling molecules, first identified by its osteoinductive capabilities⁴. It is important in several developmental processes, including gastrulation and odontogenesis⁵⁻⁷. In chick embryos, *Bmp4* is expressed in the presumptive neural folds of the hindbrain from as early as stage 6 (data not shown). By stage 9, the gene is expressed in the dorsal neural tube, with elevated levels of transcript being found in rhombomere r3. At this stage r3 is already depleted of neural crest cells and is associated with increased apoptosis and *msx2* expression (Fig. 1)³. High level *Bmp4* expression in the dorsal aspect of r3 is maintained for the next two stages, when a second site of strong expression becomes evident in r5, again distinguished by its high levels of apoptosis and *msx2* expression (Fig. 1). These two sites

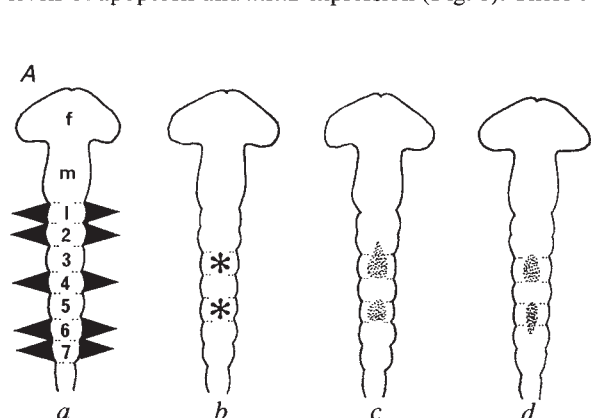


FIG. 1 A; Diagrams of a, neural crest streams emanating from the hindbrain (f, forebrain; m, midbrain); b, foci of apoptosis in rhombomeres 3 and 5 during the period of neural crest production³; c, *msx2* expression pattern during period of crest production; and d, *Bmp4* expression during period of crest production. B; *Bmp4* expression in the hindbrain during the period of crest production as revealed by whole-mount *in situ* hybridization. a, Dorsal view of a stage 9 embryo showing elevated levels of *Bmp4* transcript in r3, which at this stage is also marked by raised levels of apoptosis and *msx2* expression. b, Dorsal view of a stage-10 embryo which now has increased *Bmp4* expression in r5. As before, this is coincident with increased apoptosis and *msx2* expression.

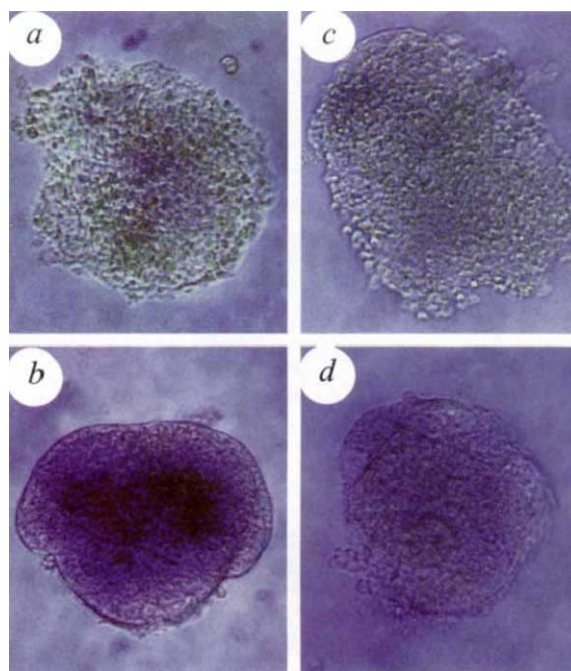
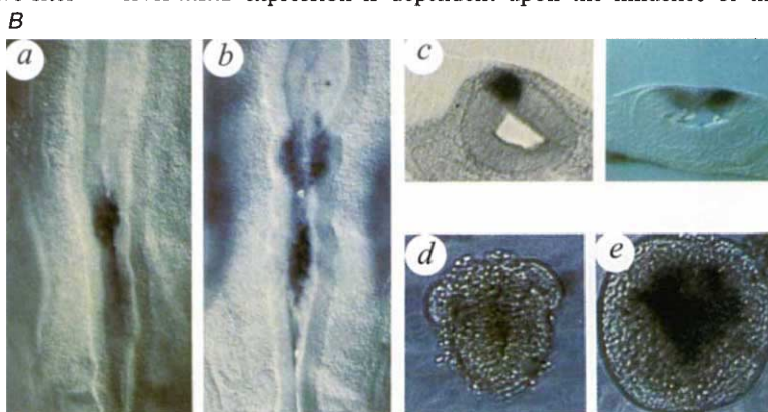


FIG. 2 BMP4 mediates upregulation of *msx2* in stage-9 explants of r3 but not of r4. a, Isolated r3 cultured in control medium does not express *msx2*. b, Addition of recombinant BMP4 at 10 ng ml⁻¹ to the medium stimulates strong *msx2* expression in r3 explants. c, Isolated r4 cultured in control medium does not express *msx2*. d, Addition of recombinant BMP4 at 10 or 100 ng ml⁻¹ does not result in *msx2* expression. METHODS. Rhombomeres were dissected as described³ and incubated for 6 h in either F-12 medium (Gibco-BRL) or F-12 with recombinant BMP4 at 1–100 ng ml⁻¹. Explants were then fixed and immobilized for *in situ* hybridization as for Fig. 1.

of *Bmp4* expression are restricted to the dorsal cells of the neural tube, which would encompass the neural crest primordium, and colocalize with both the expression domains of *msx2* and the foci of apoptosis. We have previously demonstrated that high level *msx2* expression is dependent upon the influence of the



c, Transverse 50- μ m vibratome sections through r3 and r5 at stage 9 showing *Bmp4* expression restricted to the dorsal aspect of the neural tube, in the region where crest cells would be expected to emerge. d, r3 isolated at stage 9 and cultured for 6 h does not express *Bmp4*. e, High-level *Bmp4* expression is maintained in the r3 explant when it is explanted conjoined with r4. METHODS. Whole-mount digoxigenin *in situ* hybridization was used to analyse expression patterns of *Bmp4*. After 6 h, explants were fixed in 4% paraformaldehyde and immobilized in a matrix of 8% gelatin to allow handling during the *in situ* procedure³. The *Bmp4* probe is described in ref. 11; stages are those described in ref. 12.

neighbouring rhombomeres³. This is also the case for *Bmp4*. When r3 is explanted into culture and then fixed after six hours, almost no *Bmp4* expression can be detected. By contrast, explants combining r3 with r4 maintain both the high level of *Bmp4* transcripts in r3 and the 'Y'-shaped domain configuration which is normally associated with *Bmp4* expression in r3 at stage 11 (Fig. 1A, B).

We have analysed the role of BMP4 in the control of *msx2* expression. Consistent with our previous results, explants of r3 cultured in isolation barely express *msx2* (Fig. 2). But when recombinant BMP4 is added to the medium at 10 ng ml⁻¹, strong expression of *msx2* becomes evident (Fig. 2). By contrast, application of BMP4 to r4 explants does not induce expression of *msx2* (Fig. 2), demonstrating that BMP4 is acting specifically to stimulate *msx2* expression in r3, which normally expresses it *in ovo*.

The expression pattern of *Bmp4* is consistent with its playing a role in the depletion of neural crest from rhombomeres 3 and 5. These areas are normally cleared of neural crest by an interaction with their neighbouring even-numbered rhombomeres³. When r3 or r5 are isolated from their neighbours, either by explant culture or by heterotopic transplantation, they become unconstrained and produce many neural crest cells. We have tested whether BMP4 participates in the process of crest depletion by adding recombinant protein to isolated rhombomere

explants over a range of concentrations and assessing the extent of neural crest cell production. Explanted r4 normally produces a vast quantity of neural crest when cultured on a fibronectin substrate in control medium. Neural crest production from r4 is unaffected by the addition of recombinant BMP4 up to 100 ng ml⁻¹ (Fig. 3). By contrast, explants of isolated r3 or r5 are severely crest-depleted when recombinant BMP4 is added to the cultures at concentrations as low as 1 ng ml⁻¹ (Fig. 3). At 0.1 ng ml⁻¹, production of crest from both odd rhombomeres remains evident, with the amount of crest emerging from r5 being indistinguishable from untreated explants. The effect of BMP4 treatment on the production of crest by r3 and r5 is mimicked by the closely related molecule BMP2 (98% amino-acid sequence identity)⁴, over the same concentration range (data not shown). Although other members of the TGF- β superfamily, such as *dorsalin-1* and TGF- β 2, affect trunk neural crest production and emigration^{8,9}, treatment of explanted rhombomeres with these factors, even at doses as high as 100 ng ml⁻¹, did not alter crest production (data not shown).

Our previous studies suggested that depletion of neural crest cells from rhombomeres 3 and 5 is due to the induction of apoptosis in these populations. We have tested whether the BMP4-sponsored depletion of neural crest from rhombomere 3 is due to its acting through the induction of programmed cell death. We analysed the extent of cell death, using Nile-blue

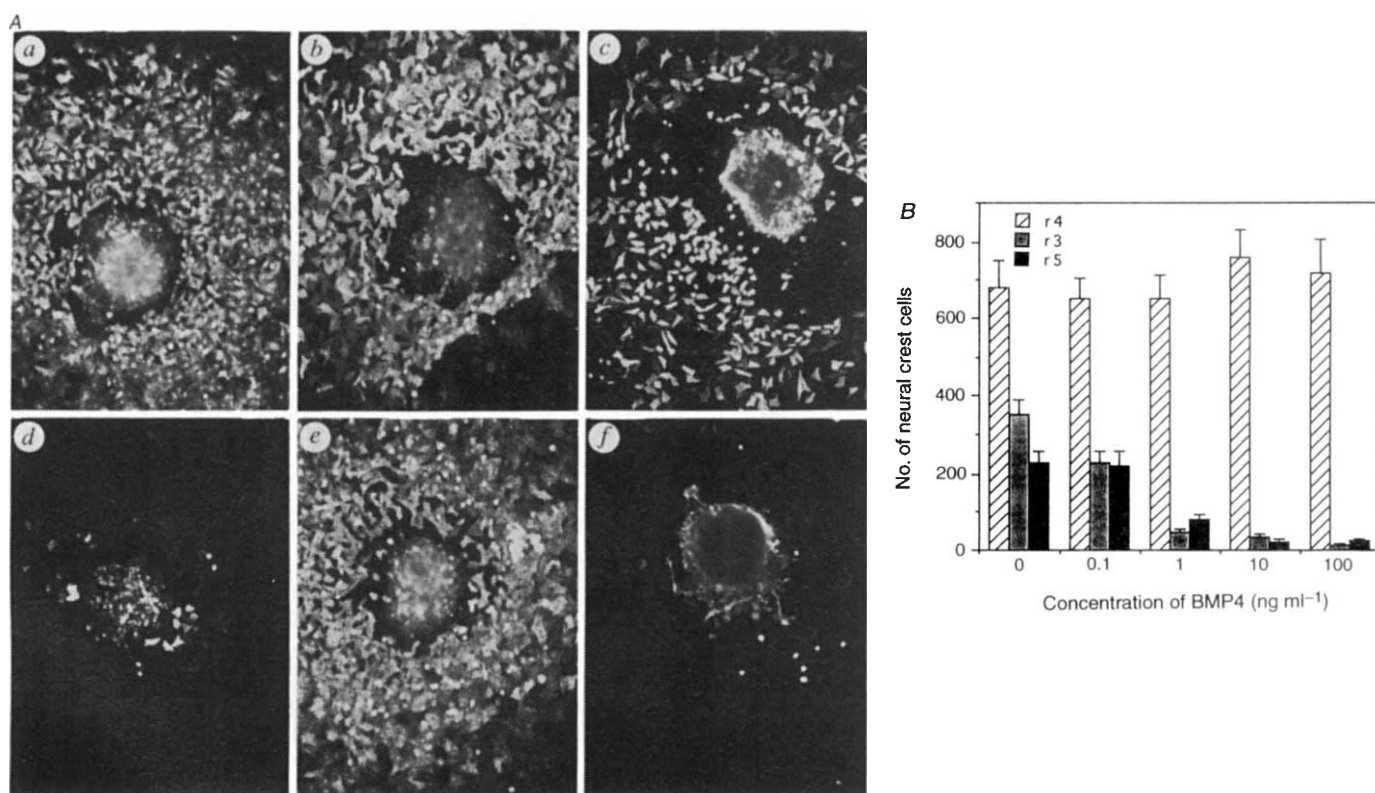


FIG. 3 BMP4 depletes crest from isolated r3 and r5 but not from isolated r4. A, Explants of isolated rhombomeres 3, 4 or 5 generate extensive crest cell outgrowth when cultured in F-12 medium (a, b, c). Addition of recombinant BMP4 (10 ng ml⁻¹) to the medium results in depletion of neural crest from r3 (d) and r5 (f) but not from r4 (e). B, Graph of the effect of recombinant BMP4 at a range of concentrations on the production of neural crest from rhombomeres 3, 4 and 5. Standard errors are indicated. Numbers of cases for each concentration are as follows (in order r3, r4, r5): 0 ng ml⁻¹, 10, 6, 9; 0.1 ng ml⁻¹, 8, 8, 6; 1 ng ml⁻¹, 14, 5, 11; 10 ng ml⁻¹, 15, 5, 10; 100 ng ml⁻¹, 17, 5, 10. METHODS. Rhombomeres were dissected and explanted as described in ref. 3 except that in these experiments the explants were plated onto 96-well tissue-culture plates (Nunc) that had been coated overnight at

4 °C with a 10 μ g ml⁻¹ solution of fibronectin in F-12 (Gibco-BRL). Explants were then incubated for 24 h either in F-12 alone or in F-12 containing recombinant BMP4. Formaldehyde was added to 3.5% to fix the explants. To visualize neural crest cells, fixed cultures were then stained with an antibody against the HNK-1 epitope (Leu-7, Becton-Dickinson) at a dilution of 1:100 in PBS/5% FCS/1% Triton overnight at 4 °C. This was followed by a 1-h incubation with an FITC-conjugated anti-mouse Ig antibody. Specimens were viewed by laser-scanning confocal microscopy (BioRad MRC 600) and by phase-contrast microscopy. To follow specifically the neural crest population of explants cultured F-12 or in BMP4, we prelabelled rhombomeres before dissection as described³.

sulphate staining³, in explants of isolated rhombomeres cultured in control medium or after treatment with BMP4 (Fig. 4). Although there are a few scattered dead cells in all cases when rhombomere 3 is treated with BMP4, a focus of intensely staining cells is evident (Fig. 4). We also used transmission electron microscopy to examine the morphology of the dying cells. We find that in explants of rhombomere 3 treated with BMP4 there is a localized region of dead or dying cells which have morphological features typical of apoptosis (Fig. 4B).

We have previously demonstrated that the apoptotic elimination of neural crest cells in rhombomeres 3 and 5 is effected by an interaction with the neighbouring even-numbered rhombomeres. The results presented here are consistent with that effect being elicited through the induction of high-level *Bmp4* expression in the neural crest primordium of rhombomeres 3 and 5. Thus *Bmp4* can be seen to be pivotal in this process, acting to stimulate *msx2* expression and the depletion of neural crest cells from

rhombomeres 3 and 5 through the induction of apoptosis. These observations also strengthen the association between *msx2* expression and morphogenetic cell death in the rhombencephalon at these early stages of development. Thus, it is not the direct action of even-numbered rhombomeres upon rhombomeres 3 and 5 that effects the cell death programme, rather this is carried out by the induction of *Bmp4* in rhombomeres 3 and 5 themselves. We have also demonstrated that *Bmp4* will cause the depletion of neural crest from isolated r3 and r5 but not from r4. This suggests that there are intrinsic differences between rhombomeres 3 and 5 and rhombomere 4, possibly in the form of the deployment of as-yet undefined vertebrate BMP4 receptors. The control of cell death by BMP4 in this system also appears to involve an autocrine loop in which *Bmp4* expression depends upon BMP4 (ref. 7). This raises the possibility that cells expressing *Bmp4* are then eliminated by it in a manner analogous to autocrine survival function of brain-derived neurotrophic factor in dorsal root ganglion cells¹⁰. This work provides further evidence to support the idea that the sculpting of neural crest into discrete streams which will populate and pattern the branchial arches is achieved by a mechanism intrinsic to the neuro-epithelium. □

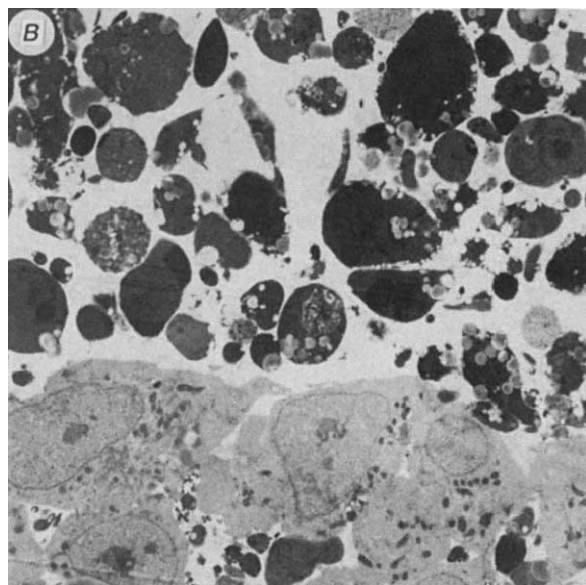
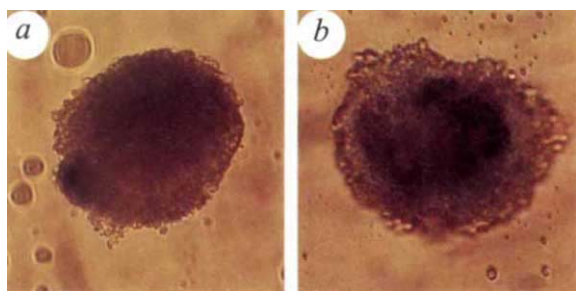


FIG. 4 BMP4 effects depletion of neural crest cells from r3 and r5 through stimulation of apoptosis. A, Assessment of cell death in explants using Nile blue sulphate staining. a, Rhombomere 3 cultured in F-12 stains weakly; and b, treatment of r3 with 10 ng ml^{-1} BMP4 induces programmed cell death, as indicated by strong localized Nile blue sulphate staining. Little staining is evident in r4 cultured in either F-12 or in BMP4 at 10 ng ml^{-1} (data not shown). B, Transmission electron micrograph of the foci of dead cells present in r3 after BMP4 treatment at 10 ng ml^{-1} , showing cells displaying features typical of apoptosis such as pyknotic nuclei with condensed chromatin.

METHODS. Rhombomeres were dissected out³ and incubated for 6 h in either F-12 or F-12 with recombinant BMP4 at 10 ng ml^{-1} . Explants were incubated in 4-well dishes (Nunc) which were first coated with 0.33% agarose to prevent explants adhering. Explants were stained with Nile blue sulphate ($1:133,000$ dilution³) and incubated for 10 min at 37°C , washed and photographed.

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Formation and inactivation of endogenous cannabinoid anandamide in central neurons

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ANANDAMIDE (*N*-arachidonoyl-ethanolamine) was recently identified as a brain arachidonate derivative that binds to and activates cannabinoid receptors^{1–4}, yet the mechanisms underlying formation, release and inactivation of this putative messenger molecule are still unclear. Here we report that anandamide is produced in and released from cultured brain neurons in a calcium ion-dependent manner when the neurons are stimulated with membrane-depolarizing agents. Anandamide formation occurs through phosphodiesterase-mediated cleavage of a novel phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine. A similar mechanism

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also governs the formation of a family of anandamide congeners, whose possible roles in neuronal signalling remain unknown. Our results and those of others^{5,6} indicate therefore that multiple biochemical pathways may participate in anandamide formation in brain tissue. The life span of extracellular anandamide is limited by a rapid and selective process of cellular uptake, which is accompanied by hydrolytic degradation to ethanolamine and arachidonate. Our results thus strongly support the proposed role of anandamide as an endogenous neuronal messenger.

We studied anandamide formation in primary cultures of rat brain striatal or cortical neurons, labelled with [³H]ethanolamine and stimulated with the Ca²⁺ ionophore, ionomycin, or with various membrane-depolarizing agents. Ionomycin stimulates formation of a radioactive component coeluting with anandamide on high-performance liquid chromatography (HPLC) (Fig. 1a–c). A similar stimulation is produced by high K⁺, kainate (a glutamate-receptor agonist) and 4-aminopyridine (a K⁺ channel blocker) (Fig. 1c). Chelating extracellular Ca²⁺ with EGTA prevents the effect of high K⁺ suggesting that anandamide formation is Ca²⁺ dependent (Fig. 1c).

By analogy with neurotransmitters and various lipid mediators^{7–9}, anandamide is expected to have to exit neurons to activate cannabinoid receptors on neighbouring cells. We find, however, that only ~20% of the radioactive material in anandamide fractions is recovered in the medium after stimulation with ionomycin, whereas ~80% remains associated with cells (Fig. 1d). Because neurons and glia rapidly take up extracellular anandamide (see below), to demonstrate anandamide release we stimulated neurons with ionomycin in the presence of exogenous anandamide (which saturates the uptake system, see below) or bovine serum albumin (which traps hydrophobic compounds). Both treatments increase anandamide-associated radioactivity in the medium and decrease it within cells, suggesting that anandamide is released and subsequently recaptured by cells (Fig. 1d). The radioactive material coeluting with anandamide is further resolved by HPLC into several components, which coelute with the following *N*-acyl-ethanolamines (NAE): (1) *N*- γ -linolenoyl-; (2) *N*-arachidonoyl- (=anandamide) plus *N*-linoleoyl-; (3) *N*-palmitoyl-; (4) *N*-oleoyl-; and (5) *N*-stearoyl-ethanolamine (Fig. 1e). Additional HPLC analysis allowed us to resolve component 2 into two products coeluting with anandamide (~80%) and *N*-linoleoyl-ethanolamine (~20%). This identification was confirmed by gas chromatography/mass spectrometry (GC/MS). From the mass spectrum of synthetic anandamide (Fig. 1f), we chose characteristic ions for mass fragmentography. Analysis of stimulated neurons reveals that a product is eluted from the GC at the retention time of anandamide (~0.5 pmol per dish with ionomycin and ~2 pmol per dish with kainate). Fragmentation of this product yields ions characteristic of anandamide, at *m/z* 329, 244, 145 and 85 (Fig. 1f, *g*₍₁₎ and data not shown). GC elution and MS fragmentation confirm that stimulated neurons produce *N*-linoleoyl-, *N*-oleoyl-, *N*-stearoyl- and *N*-palmitoyl-ethanolamine (Fig. 1g_{(2)–(5)}). Ions characteristic of *N*- γ -linolenoyl-ethanolamine were not detectable and identification of this product must therefore be tentative.

To determine whether striatal or cortical astroglia contribute to anandamide formation, we stimulated neuron-free cultures of astrocytes¹⁰ with ionomycin after [³H]ethanolamine labelling. We found no radioactivity coeluting with anandamide, indicating that formation occurs in neurons (control, 82 ± 34 d.p.m. per dish; ionomycin, 89 ± 26 d.p.m. per dish, background, 43 ± 3 d.p.m. per dish, *n* = 3).

Previous studies on NAE suggest two possible mechanisms of anandamide formation: (1) energy-independent condensation of free arachidonate with ethanolamine^{5,6,11–13}, or (2) release by phospholipase D-mediated cleavage of the phospholipid precursor, *N*-arachidonoyl phosphatidylethanolamine (PE) (Fig. 2). This phospholipid has not yet been isolated from mammalian tissues, but the occurrence of related *N*-acyl PEs has been demonstrated^{14–16}.

Because levels of free ethanolamine and arachidonate in resting neurons are low^{7,16}, for condensation to occur multiple enzyme activities should be stimulated concomitantly: phospholipase A₂ (PLA₂) or phospholipase C (PLC) (to generate arachidonate) and phospholipase D (PLD) (to generate ethanolamine)^{5,6}. We find that two selective PLA₂ inhibitors, dimethyleicosadienoic acid and octadecylbenzoic acid, and the PLC inhibitor, neomycin, have no effect on ionomycin-stimulated anandamide formation. Moreover, incubation of intact neurons with exogenous PLD (*Streptomyces chromofuscus*, 40 units ml⁻¹, 10 min), but not PLC or PLA₂, is sufficient to produce anandamide and other NAE. Despite the large quantities of ethanolamine released by exogenous PLD, addition of arachidonate (60 μ M) does not increase anandamide formation, indicating that condensation does not occur in the intact neurons (data not shown).

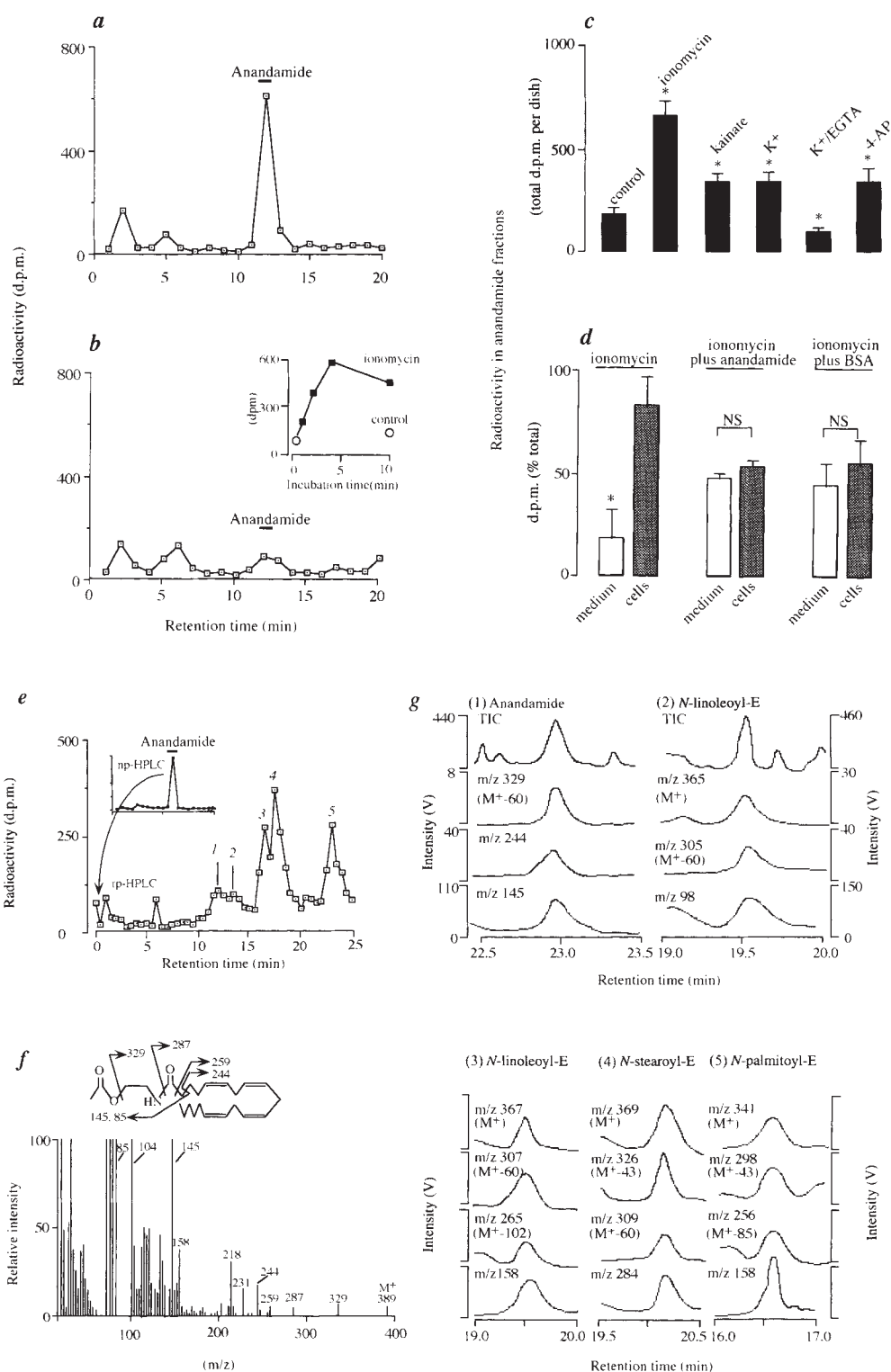
These results argue against a role for energy-independent condensation in anandamide formation and suggest that a phospholipid precursor of anandamide and other NAE exists in neurons. Because *N*-arachidonoyl PE and other *N*-acyl PE meet the structural criteria for such a precursor (Fig. 2), we purified *N*-acyl PE from cultured neurons, and identified it by thin-layer chromatography, HPLC, enzymatic digestion and proton nuclear magnetic resonance spectroscopy. When purified *N*-acyl PE is digested with *S. chromofuscus* PLD, the spectrum of NAE formed (determined by HPLC and GC/MS) is qualitatively similar to that produced by ionomycin and comprises anandamide (H.C., S. Gaillet, V.D.M. and D.P., manuscript in preparation). These results indicate that *N*-acyl PE is present in neurons and is composed of multiple molecular species which include *N*-arachidonoyl PE.

To obtain direct evidence that *N*-acyl PE serves as a precursor for anandamide and other NAE, we determined whether there is in neurons a phosphodiesterase activity that hydrolyses this phospholipid. Neuron homogenates convert purified [³H]*N*-acyl PE or synthetic [³H]*N*-arachidonoyl PE into anandamide and other NAE, and this reaction is inhibited by boiling (Fig. 3a). Although we have not characterized the phosphodiesterase activity involved in *N*-arachidonoyl PE hydrolysis, a PLD activity that hydrolyses *N*-acyl PE forming NAE has been described^{17,18}.

If *N*-acyl PE served as precursor for anandamide and other NAE, stimuli that enhance formation of these metabolites should also increase *N*-acyl PE turnover. To test this, we incubated neurons with [³H]ethanolamine for 20 min, and measured [³H]*N*-acyl PE. Ionomycin produces a 2.6-fold enhancement of [³H]ethanolamine incorporation into *N*-acyl PE, without affecting [³H]PE, indicating that *N*-acyl PE turnover is selectively enhanced (Fig. 3b, c).

Next, we determined whether mechanisms exist in neural cells for the inactivation of anandamide. Exogenous [³H]anandamide is rapidly cleared from the media of neuronal cultures (Fig. 4a). This clearance is accompanied by association of [³H]anandamide with cells, a process that fulfills several criteria of a carrier-mediated uptake. [³H]Anandamide association with cells is: (1) rapid (*t*_{1/2} = 2.5 min) (Fig. 4b); (2) temperature-dependent (Fig. 4b); (3) saturable at 37 °C (Fig. 4c), and consequently reduced in a concentration-dependent manner by the addition of nonradioactive anandamide (Fig. 4d); (4) selective. [³H]*N*-palmitoyl-ethanolamine is not recaptured (Fig. 4a) and other NAE do not compete for [³H]anandamide uptake (Fig. 4d). Uptake of [³H]ethanolamine-labelled anandamide is accompanied by formation of free [³H]ethanolamine and, to a minor extent, [³H]PE (Fig. 4e, f). Moreover, when [³H]arachidonate-labelled anandamide is used, radioactivity is recovered in all phospholipid classes, but not in free arachidonate (Fig. 4e, f). These results suggest that an endogenous amidohydrolase activity^{11,17} catalyses the cleavage of anandamide to ethanolamine and arachidonate, which are then incorporated into phospholipids (Fig. 2). Astrocytes in culture take up and degrade [³H]anandamide to a degree similar to neurons,

FIG. 1 Formation and release of anandamide from neurons in primary culture. **a–c**, In rat striatal neurons labelled by incubation with [3 H]ethanolamine, ionomycin and depolarizing stimuli evoke formation of radioactive material eluting at the retention time of anandamide. Representative normal-phase (np)HPLC fractionation of samples from the 10-min incubation of neurons (one dish) **a**, with ionomycin (1 μ M) and **b**, without ionomycin. Bars indicate the retention time of synthetic anandamide. The inset in **b** depicts the time-course of the ionomycin effect. **c**, Effects of ionomycin, kainate (200 μ M), KCl (40 mM) and 4-aminopyridine (4-AP, 3 mM) on the formation of radioactive material in anandamide np-HPLC fractions. Results represent the mean $\pm$ s.e.m. of 3–5 experiments, each done on one dish of neuronal cultures. *, $P < 0.05$ (ANOVA). **d**, Release from striatal neurons of radioactive material in anandamide fractions. Incubations were in culture medium containing either exogenous anandamide (100 μ M) or fatty acid-free bovine serum albumin (BSA, 0.5%). Results represent the mean $\pm$ s.e.m. of 3–5 experiments. * Statistically different, or not significant (NS), $P < 0.05$ (Student's *t*-test). **e**, HPLC identification of anandamide and related *N*-acyl-ethanolamines formed in ionomycin-stimulated cortical neurons. Representative np- and rp-HPLC fractionations of samples from the 10-min incubation (four dishes) with ionomycin of neurons labelled with [3 H]ethanolamine. Numbers indicate the retention times of the following synthetic *N*-acyl-ethanolamines: (1) *N*- γ -linolenoyl-ethanolamine; (2) *N*-linoleoyl-ethanolamine plus anandamide; (3) *N*-palmitoyl-ethanolamine; (4) *N*-oleoyl-ethanolamine; (5) *N*-stearoyl-ethanolamine. **f, g**, GC/MS identification of anandamide and related *N*-acyl-ethanolamines. **f**, Electron-impact mass spectrum (70 eV) of synthetic anandamide (O-acetyl derivative). **g**, Mass fragmentographic analyses of anandamide and related *N*-acyl-ethanolamines (*N*-acyl-E) produced by cortical neurons stimulated with ionomycin. (1) Anandamide; (2) *N*-linoleoyl-E; (3) *N*-oleoyl-E; (4) *N*-stearoyl-E; (5) *N*-palmitoyl-E. Intensities are omitted from (3)–(5) for clarity, but the relative abundances of the diagnostic ions correspond to those obtained with synthetic standards (A.F., V.D.M., H.C. and D.P., manuscript in preparation). TIC, total ion current.



(16–20 h) with [3 H]ethanolamine (1 μ Ci ml⁻¹, 34 Ci mmol⁻¹, Amersham), were washed with Dulbecco's modified Eagle's medium (DMEM) and incubated for various times in DMEM (5 ml) containing drugs at the indicated concentrations. Incubations were stopped by adding methanol, immediately followed by chloroform extraction. Fractionation and analyses of anandamide and related *N*-acyl-ethanolamines will be described in detail elsewhere (A.F., V.D.M., H.C. and D.P., manuscript in preparation). Chemical syntheses of *N*-acyl-ethanolamines used as standards were as described¹. Identities of the synthetic products were verified by proton nuclear magnetic resonance spectroscopy (NMR) and by GC/MS, after purification of the products by HPLC.

indicating that glia contribute to [^3H]anandamide disposition (data not shown).

Our results show that brain neurons produce, release and inactivate anandamide, strongly supporting a role for this arachidonate derivative as an endogenous cannabinoid substance. They also indicate that anandamide, unlike neurotransmitters,

neuropeptides and eicosanoids, may be produced through a single-step, phosphodiesterase-mediated cleavage of a novel phospholipid, *N*-arachidonoyl PE (Fig. 2), and that a family of chemically related NAE may be coreleased through a similar mechanism. The biological roles of the NAE remain unknown, but their pharmacological effects on excitable tissues suggest that

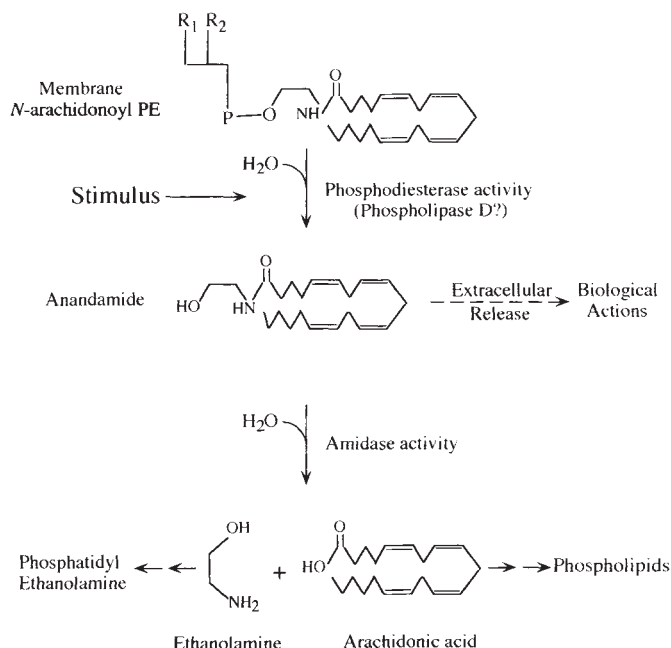


FIG. 2 Proposed pathway for the formation and inactivation of anandamide in brain neurons. According to this model, stimuli (such as increased intracellular Ca^{2+}) evoke the hydrolytic cleavage of a phospholipid precursor in neuronal membranes, *N*-arachidonoyl phosphatidylethanolamine (*N*-arachidonoyl PE). Hydrolysis of *N*-arachidonoyl PE may be catalysed by a D-type phosphodiesterase activity (phospholipase D), producing anandamide in a single-step reaction, or by C-type phosphodiesterase activity (phospholipase C), producing a phosphorylated form of anandamide which would be converted to anandamide by phosphatase activity. The family of *N*-acyl-ethanolamines described in this study are probably generated from *N*-acyl PE through a similar mechanism. An additional mechanism of anandamide formation, the enzymatic condensation of arachidonate with ethanolamine, was also proposed^{5,6}. Newly generated anandamide may exit neurons to activate receptors on target cells. The biological actions of anandamide may be terminated by two concurrent processes, cellular uptake (not shown) and hydrolytic degradation to arachidonate and ethanolamine, which are both incorporated into cellular phospholipids.

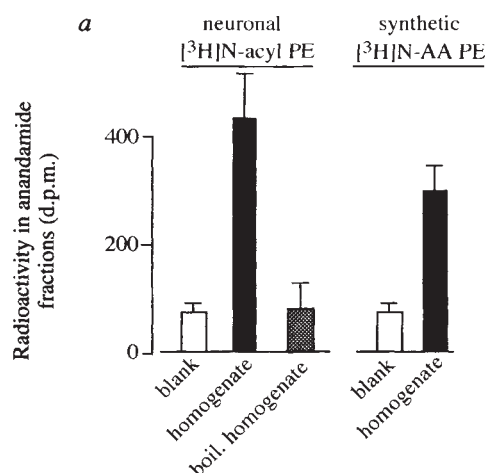
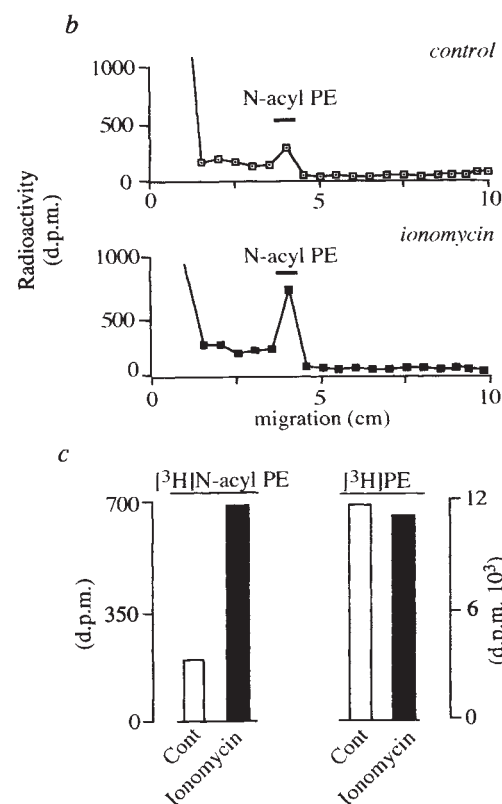


FIG. 3 Enzymatic hydrolysis and ionomycin-evoked turnover of *N*-acyl PE. **a**, Hydrolysis of purified [^3H]N-acyl PE and synthetic [^3H]N-arachidonoyl PE by neuron homogenates. Results present the mean $\pm$ s.e.m. of 3–7 experiments. **b**, Effect of ionomycin on the labelling of *N*-acyl PE. Representative TLC fractionation of samples from a 20-min incubation with [^3H]ethanolamine, alone (top), or together with ionomycin (1 μM , bottom). **c**, Effects of ionomycin on the labelling of *N*-acyl PE with [^3H]ethanolamine. Results are from the experiment shown in **b**. Similar results were obtained in two additional experiments.

METHODS. Neuronal [^3H]N-acyl PE was fractionated by silica gel G column chromatography followed by TLC using a solvent system of chloroform, methanol, ammonium hydroxide (80/20/1, vol/vol/vol). Neuron homogenates were incubated for 10 min at 37 $^{\circ}\text{C}$ in Tris buffer (25 mM, pH 7, 0.4 ml) containing purified [^3H]N-acyl PE or synthetic [^3H]arachidonoyl PE (10,000 d.p.m.). *N*-arachidonoyl PE was synthesized by reacting arachidonic acid with *L*- α -dipalmitoyl-phosphatidylethanolamine in the presence of diisopropylcarbodiimide. Identity of synthetic *N*-arachidonoyl PE was verified, after TLC purification, by



proton nuclear magnetic resonance spectroscopy. An additional sample of *N*-arachidonoyl PE was provided by Avanti Polar Lipids Inc. (Alabaster, AL, USA). [^3H](Arachidonoyl)*N*-arachidonoyl PE, synthesized as described above using [^3H] arachidonic acid (20 μCi), was purified by TLC.

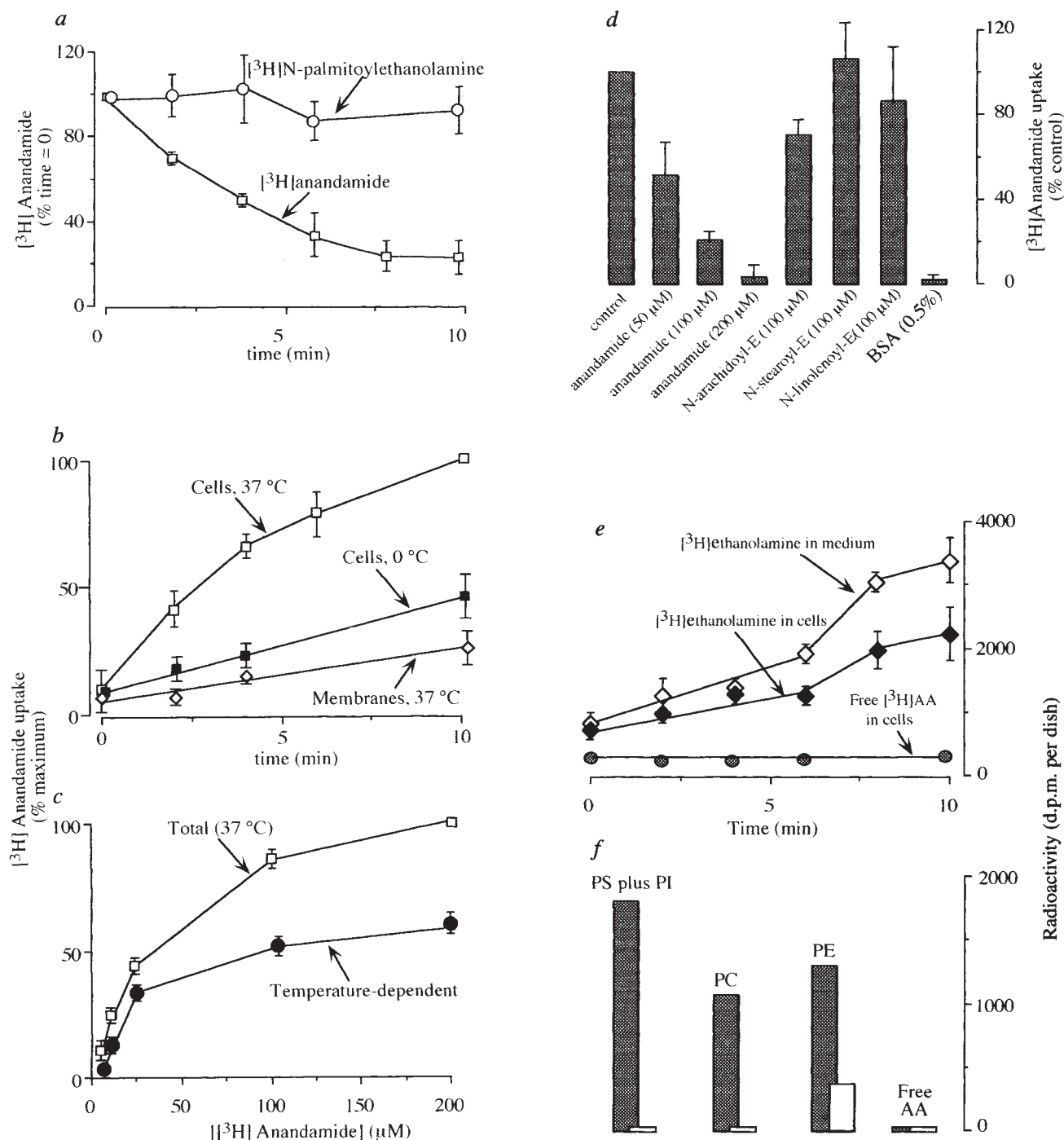


FIG. 4 Uptake and degradation of exogenous $[^3\text{H}]$ anandamide. **a**, Time-dependent clearance of $[^3\text{H}]$ anandamide from the incubation medium of cultures of cortical neurons ($\square$). $[^3\text{H}]$ N-palmitoyl-ethanolamine is not cleared from medium ($\circ$). **b**, Time-dependent association of $[^3\text{H}]$ anandamide with intact neuronal cultures at 37°C ($\square$), intact neuronal cultures at $0-4^\circ\text{C}$ ($\blacksquare$), or neuronal particulate fraction at 37°C ($\diamond$). **c**, Kinetics of $[^3\text{H}]$ anandamide association (2 min) with neuronal cultures. Temperature-dependent association ($\bullet$), calculated by subtracting association at $0-4^\circ\text{C}$ (not shown) from total association at 37°C ($\square$). **d**, Effects of anandamide, N-acyl-ethanolamines (N-acyl-E) and BSA on the temperature-dependent association of $[^3\text{H}]$ anandamide with neuronal cultures. Bars indicate the mean $\pm$ s.e.m. from 3 experiments. **e**, **f**, Hydrolytic cleavage of $[^3\text{H}]$ anandamide to $[^3\text{H}]$ ethanolamine and $[^3\text{H}]$ arachidonate. **e**, Accumulation of $[^3\text{H}]$ ethanolamine inside ($\blacklozenge$) and outside ($\diamond$) cultures incubated with $[^3\text{H}]$ anandamide, labelled on either the ethanolamine or the arachidonate moiety. Free $[^3\text{H}]$ arachidonate (AA) does not accumulate under these conditions

(shaded circle). **f**, Accumulation of $[^3\text{H}]$ arachidonate (filled bars) or $[^3\text{H}]$ ethanolamine (empty bars) in phospholipids of neuronal cultures incubated with $[^3\text{H}]$ anandamide. AA, Arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. **METHODS.** Neuronal cultures were incubated at 37°C or $0-4^\circ\text{C}$ in DMEM containing $[^3\text{H}]$ anandamide ($6.25-200\ \mu\text{M}$, $0.3\ \text{mCi}\ \text{mmol}^{-1}$) or $[^3\text{H}]$ N-palmitoyl-ethanolamine ($10\ \mu\text{M}$, $3.5\ \text{mCi}\ \text{mmol}^{-1}$). Samples of media were taken at the indicated times, and extracted immediately with methanol/chloroform. Cells were washed with repeated applications of DMEM containing BSA (0.5%) before extraction. $[^3\text{H}]$ anandamide and $[^3\text{H}]$ N-palmitoyl-ethanolamine were measured in the lipid extracts by silica gel G column chromatography. In preliminary experiments, $[^3\text{H}]$ anandamide was measured by HPLC, with identical results. $[^3\text{H}]$ Ethanolamine was determined as detailed elsewhere (F. Desarnaud, H.C. and D.P., manuscript in preparation).

they may participate in cellular signalling^{19,20}. Anandamide may be also synthesized by condensation of arachidonate with ethanolamine, a reaction catalysed by NAE amidohydrolase acting in reverse¹¹ or by anandamide synthetase^{3,6}. Although our results may not be accounted for by such mechanism, they do not rule out its participation in anandamide formation under different conditions. It is possible for instance that, by analogy with platelet-activating factor²¹, distinct physiological stimuli may cause anandamide formation by activating distinct biochemical pathways. □

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Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells

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MAJOR histocompatibility complex (MHC) class I and class II molecules bind immunogenic peptides and present them to lymphocytes bearing the $\alpha\beta$ T-cell antigen receptor (TCR)^{1–4}. An analogous antigen-presenting function also has been proposed for the non-MHC-encoded CD1 molecules⁵, a family of non-polymorphic, β_2 -microglobulin-associated glycoproteins^{5–8} expressed on most professional antigen-presenting cells^{9–11}. In support of this hypothesis, CD1 molecules are recognized by selected CD4⁺CD8[−] $\alpha\beta$ or $\gamma\delta$ TCR⁺ T-cell clones^{12–14}, and we have recently shown that CD1 molecules restrict the recognition of foreign microbial antigens by $\alpha\beta$ TCR⁺ T cells¹⁰. But the substantial structural divergence of CD1 from MHC class I and class II molecules⁷, raises the possibility that the antigens presented by the CD1 system may differ fundamentally from those presented by MHC-encoded molecules. Here we report that a purified CD1b-restricted antigen of *Mycobacterium tuberculosis* presented to $\alpha\beta$ TCR⁺ T cells is mycolic acid, a family of α -branched, β -hydroxy, long-chain fatty

acids found in mycobacteria^{15,16}. This example of non-protein microbial antigen recognition suggests that $\alpha\beta$ TCR⁺ T cells recognize a broader range of antigens than previously appreciated and that at least one member of the CD1 family has evolved the ability to present lipid antigens.

We have previously described the CD1b-restricted recognition of an exogenous antigen contained in sonicated extracts of *M. tuberculosis* by the CD4⁺CD8[−] $\alpha\beta$ TCR⁺ T-cell line DN1. Additional studies demonstrated a requirement for a chloroquine-sensitive antigen-processing step which was similar, but not identical to that required by MHC class II¹⁰. However, in contrast to MHC class II-restricted protein antigens, an initial assessment of the chemical nature of the CD1b-restricted mycobacterial antigen revealed that the antigen was resistant to protease treatment (Fig. 1).

Further studies using detergents and cell fractionation methods suggested that the CD1b-restricted antigen was a hydrophobic molecule associated with the bacterial cell wall. Given that mycobacteria contain a large variety of unique lipids as integral components of their cell walls^{15,16}, we consid-

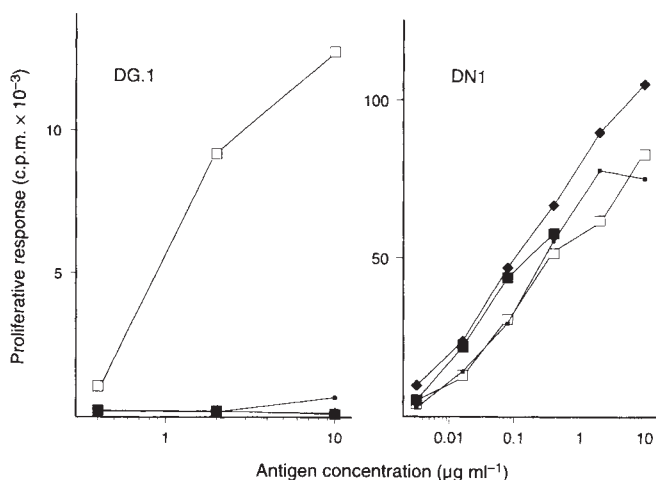


FIG. 1 Protease resistance of CD1b-restricted antigen recognition. The *M. tuberculosis* antigen preparation was digested with proteases having either limited amino-acid specificity (trypsin type XIII (Sigma) (Lys, Arg)(◆), V8 protease type XIII-B (Sigma) (acidic) (■)), or broad amino-acid recognition (proteinase K (Boehringer Mannheim) (■)). Identical aliquots were used to stimulate the proliferation of either the CD1b-restricted DN1 cell line or a *M. tuberculosis*-specific HLA-DR7-restricted CD4⁺ T cell line DG.1 (ref. 10). Left, Proliferative responses (c.p.m. of ³H-thymidine incorporated) of the CD4⁺ T-cell line DG.1 to mock (□) or protease digestions of *M. tuberculosis*. Antigen concentration expressed as amount of *M. tuberculosis* protein (µg ml⁻¹) is shown on the x-axis. Right, Proliferative responses of the CD1b-restricted T-cell line DN1 to the identical samples.

METHODS. Proliferation assays were as previously described¹⁰. The antigen preparation used was a 50% ammonium sulphate precipitate of an aqueous sonicate of *M. tuberculosis* (strain H37Ra, Difco) prepared as described previously¹⁰ and dialysed against 0.05 M NH₄HCO₃, pH 8.1 using a 12–14K molecular weight cutoff membrane to remove any contaminating peptides. A 100 µg (protein content) sample of the *M. tuberculosis* preparation was mixed with 10 µg of appropriate enzyme in total volume of 200 µl and incubated at 37 °C. To ensure complete digestion, incubations were done for 2 h, heat-inactivated (65 °C, 5 min) and a second aliquot of enzyme added for 15 h. The proteinase K digest contained 1 mM CaCl₂ in addition to the above buffer. Antigen-presenting cells were GM-CSF plus IL-4 treated (to induce CD1 expression¹⁰) monocytes (DN1), or EBV-transformed B cells derived from an HLA-DR7⁺ individual (DG.1). In experiments not shown, digestions with other broadly reactive proteases (pronase, subtilisin) or sequential digestions with V-8 protease, pronase and subtilisin, also had no effect on the CD1b-restricted mycobacterial antigen.

ered the possibility that the protease-resistant CD1b-restricted antigen of mycobacteria was a lipid. Thus, mycobacterial sonicates were extracted with chloroform/methanol¹⁷, and each of the resulting phases (organic, aqueous and interface) was tested by T-cell proliferation assay for antigenic activity. Strikingly, the CD1b-restricted antigen was found quantitatively in the organic phase, with the mycobacterial lipids (Fig. 2). In contrast, a conventional MHC class II-restricted polypeptide antigen was located within the protein-containing interface, and the small polar non-peptide antigen recognized by the major subset of $\gamma\delta$ TCR⁺ T cells ($V\gamma 2V\delta 2^+$)^{18,19} partitioned into the aqueous phase which held mainly polar molecules. These strongly hydrophobic characteristics of the CD1b-restricted antigen provided further evidence that it might be a bacterial lipid.

After extraction into chloroform/methanol, the CD1b-restricted mycobacterial antigen was further purified by passage over cyano-bonded silica solid-phase extraction columns (CN-SPE, JT Baker, Phillipsburg, NJ). The starting antigenic activity was fully recovered from the CN-SPE column in a fraction eluted with 85% chloroform in hexane, as determined by assaying each column fraction using the proliferative responses of line DN1. This active fraction was analysed by thin layer chromatography (TLC)²⁰ and cyano column high-pressure liquid chromatography (HPLC) which revealed that it contained only two species, free fatty acids and mycolic acids. To separate the fatty acids (typically 8–24 carbons in chain length) from the much larger mycolic acids (70–90 carbons in chain length), we used C18 reverse-phase (rp) HPLC²¹. Using a methylene chloride gradient, we found that the CD1b-restricted antigen recognized by line DN1 was coeluted with mycolic acids, which strongly suggested that the CD1b-restricted antigen was a mycolic acid (Fig. 3a).

To confirm the chromatographic data, independent sources of purified mycolic acid were tested for antigenic activity. We obtained purified samples of 6,6'-trehalose dimycolate (cord factor); one from *M. tuberculosis* (Sigma) and another from *Mycobacterium kansasii* and prepared free mycolic acids by saponification of each specimen in methanolic potassium hydroxide. As a control, fatty acids were prepared from 6,6'-trehalose dibehenate (Sigma), a synthetic cord factor that contains two C₂₂ fatty-acid chains rather than mycolic acid. The free mycolic-acid-containing fraction prepared from both purified 6,6'-trehalose dimycolate sources was able to stimulate the

T-cell line DN1, whereas the fatty-acid-containing fraction prepared from 6,6'-trehalose dibehenate was completely inactive (Fig. 3b). This argued strongly that mycolic acid, rather than trehalose (which was present in each of the samples) or fatty acid, was the CD1b-restricted antigen. Furthermore, C18 rpHPLC analysis of the saponified 6,6'-trehalose dimycolate confirmed that the biologically active species was located in fractions corresponding to early eluting peaks of mycolic acid (Fig. 3c).

Although several immunological effects have been attributed to 6,6'-trehalose dimycolate^{22,23}, no mitogenic effect on T cells has been reported. Nevertheless, to rule out the possibility that free mycolic acid was directly mitogenic or indirectly inducing proliferation of DN1 by stimulating the antigen-presenting cells, we examined the ability of a variety of T cells (CD4⁺ $\alpha\beta$ TCR⁺, CD4⁺CD8⁺ $\alpha\beta$ TCR⁺, CD4⁺CD8⁺ $\gamma\delta$ TCR⁺) to respond to rpHPLC purified mycolic acids. Mycolic acids purified from either *M. tuberculosis* sonicates or 6,6'-trehalose dimycolate were recognized by the T-cell line DN1, but not by three mycobacteria-specific non-CD1b-restricted T-cell lines (Fig. 4a). These results indicated that the recognition of mycolic acid by the DN1 line was a specific response.

b To ensure that the recognition of purified mycolic acid by line DN1 involved CD1b restriction as previously described for the crude *M. tuberculosis* extract, we used CD1 transflectants of the C18 B-lymphoblastoid cell line. Mycolic acid prepared from purified 6,6'-trehalose dimycolate was recognized by line DN1 in cytolytic assays only when presented by CD1b⁺ transflectants, but not when presented by either CD1a⁺, CD1c⁺ or mock transflectants (Fig. 4b). Therefore, specificity and restriction, the hallmarks of TCR-mediated immune responses, characterized this CD1b-restricted response to *M. tuberculosis*.

We have shown that a CD1b-restricted antigen of mycobacteria is mycolic acid, a class of long-chain fatty acids present in mycobacteria and several related genera such as nocardia, rhodococcus and corynebacteria²⁴. Mycolic acid is located within the bacterial cell wall, covalently esterified to peptidoglycan-linked arabinogalactan polysaccharides, and also hydrophobically associated in the form of 6,6'-trehalose dimycolate^{15,24–26}. Although uniform in their α -branched, β -hydroxy structure, mycolic acids are heterogeneous with regard to chain length, number of double bonds, cyclopropyl groups, and side groups (keto-, methoxy- and epoxy-groups)^{15,16,24}. Currently, it is not known whether CD1b presents one or more

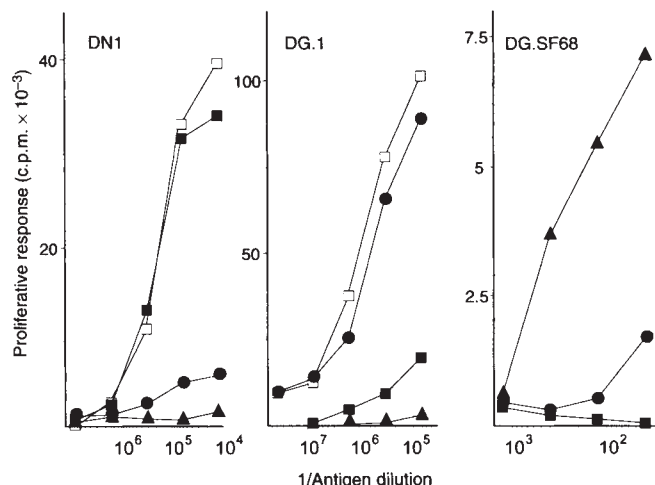


FIG. 2 Extraction of the CD1b-restricted mycobacterial antigen into organic solvents. Total mycobacterial sonicates (□) were extracted with chloroform/methanol and the resultant organic phase (■), aqueous phase (▲), and interface (●) were examined in proliferation assays. Antigen concentration along the x-axis is depicted as 1/dilution normalized to the volume of the standard sonicate preparation (see methods). Left, Proliferative response (c.p.m. of ³H-thymidine incorporated) of DN1, a CD1b-restricted, mycobacteria-specific CD4⁺CD8⁺ $\alpha\beta$ TCR⁺ T-cell line. Middle, Proliferative response of DG.1, a HLA-DR7-restricted, *M. tuberculosis*-specific CD4⁺ $\alpha\beta$ TCR⁺ T-cell line. Right, Proliferative response of DG.SF68, a *M. tuberculosis*-specific $\gamma\delta$ TCR⁺ T-cell clone. METHODS. Sonicates of *M. tuberculosis* (strain H37Ra, Difco) in PBS (200 mg bacteria per 5 ml) were extracted (4:1, v/v organic to aqueous) with chloroform:methanol (2:1, v/v) after the method of Folch¹⁷. After phase separation, fractions were dried by rotary evaporation (organic phase) or lyophilized (aqueous phase and interface) and resuspended in complete media/10% fetal calf serum by water bath sonication. Over 95% of the mycobacterial protein was found in the interface after a typical chloroform/methanol/H₂O extraction (unpublished data, E.M.B.). DG.SF68 is a $\gamma\delta$ T-cell clone expressing the V γ 2 and V δ 2 gene segments. It was derived in our laboratory by stimulation of isolated $\gamma\delta$ TCR⁺ T cells with *M. tuberculosis*¹⁹. Proliferation assays were collected on day 2 (DG.SF68), day 3 (DG.1) or day 5 (DN1) after a 6-h pulse of ³H-thymidine¹⁰. APCs were GM-CSF plus IL-4-treated CD1⁺ monocytes (DN1), HLA-DR7⁺ PBMC (DG.1) or untreated allogeneic PBMC (DG.SF68).

members of the mycolic acid family to T cells as a processed or intact acyl chain or as part of a modified epitope.

CD1 molecules show negligible homology in their $\alpha 1$ domains compared with MHC molecules and secondary structure predictions suggest that their $\alpha 2$ domain may not contain the long α -helical stretches⁷ that universally comprise the antigen-binding groove of MHC molecules. Therefore, the binding of antigen by CD1 molecules may not be strictly analogous to peptide binding by classical MHC class I and II molecules or non-classical MHC class Ib molecules. On the

basis of these differences, CD1 molecules are thought to have diverged from a common ancestral antigen-presenting gene close to the point in time that class I and II genes originated⁷. Furthermore, CD1-restricted antigen presentation occurs efficiently in T2 cells, which have deleted genes that are critical both to MHC class I and class II pathways of antigen presentation^{10,27,28}. The existence of a unique processing pathway, the divergence of CD1 structure from that of MHC molecules, and the nature of the antigens recognized by this system, indicate that CD1 antigen presentation should be

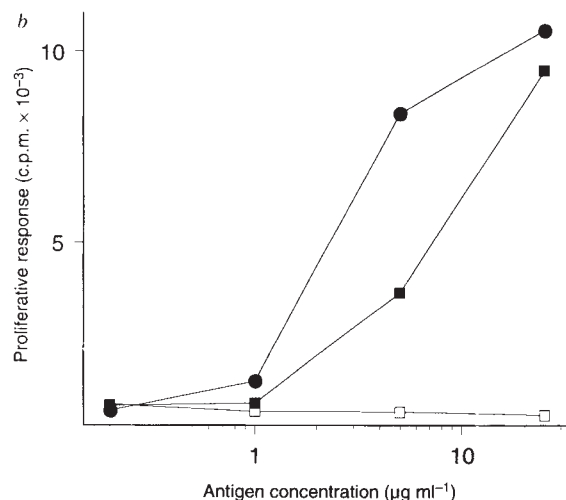
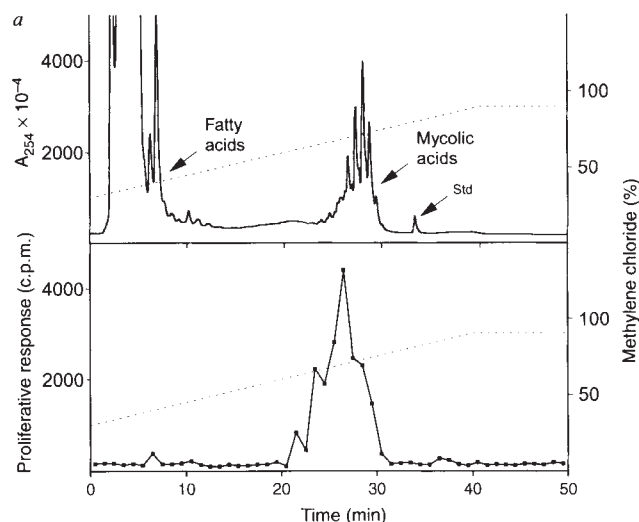
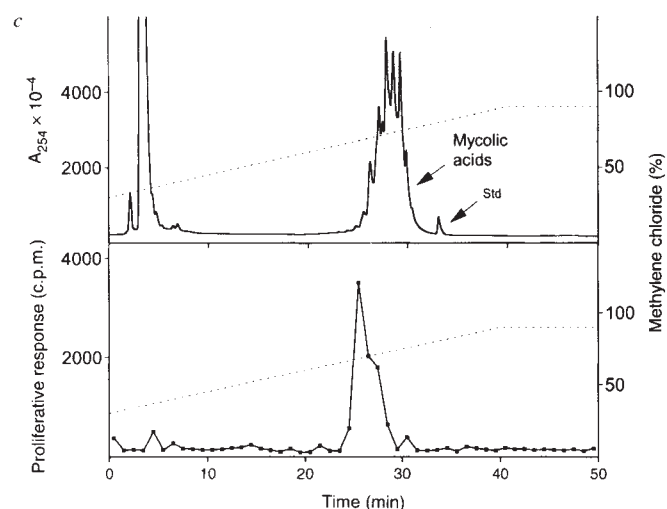


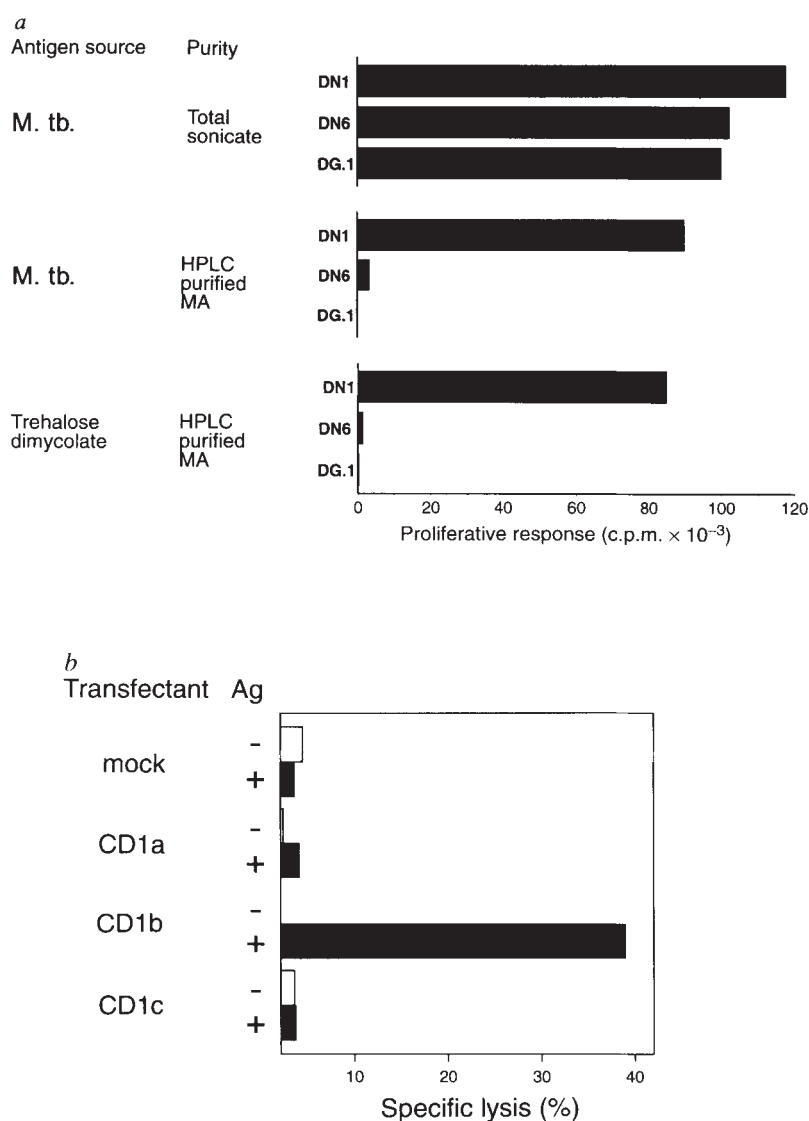
FIG. 3 Identification of the CD1b-restricted antigen as mycolic acid. *a*, Proliferative responses of the CD1b-restricted T-cell line DN1 correlated with mycolic acid peaks resolved by C18 rpHPLC from crude *M. tuberculosis* extracts. The CN-SPE column purified acyl chain fraction of *M. tuberculosis* containing the CD1b-restricted antigen (see text) was chromatographed using rpHPLC and the resulting fractions assayed for the ability to stimulate a proliferative response by T-cell line DN1. Top, Absorbance spectrum at 254 Å (solid line, expressed as absorbance units ($A \times 10^{-4}$)) of the eluted material and methylene chloride concentration (dotted line, per cent methylene chloride in methanol, v/v) of the elution gradient. Bottom, Proliferative response (in c.p.m. of ^3H -thymidine incorporated) of the T-cell line DN1 to each 1-min HPLC fraction. *b*, Saponified 6,6'-trehalose dimycolate (cord factor) but not saponified 6,6'-trehalose dibehenate stimulated a proliferative response by the CD1b-restricted T-cell line. Purified 6,6'-trehalose dimycolate from either *M. tuberculosis* (H37Ra) (■) or *M. kansasii* (●) was saponified and assayed for the ability to induce a proliferative response by line DN1. 6,6'-trehalose dibehenate (synthetic cord factor) (□) was treated identically. Antigen concentration is expressed in $\mu\text{g ml}^{-1}$ of cord factor along the x-axis. *c*, Fractions corresponding to early mycolic acid peaks from rpHPLC separation of saponified 6,6'-trehalose dimycolate (from *M. tuberculosis* (H37Ra)) stimulated the CD1b-restricted T-cell line. Mycolic acids generated by saponification of 6,6'-trehalose dimycolate from *M. tuberculosis* (Sigma) were chromatographed as in *a*, and fractions assayed for the ability to induce a proliferative response by line DN1. Both panels are as described in *a*.

METHODS. The CN-SPE column-purified mycobacterial fraction was used for rpHPLC in *a*. To obtain this fraction, the organic phase from an extraction of mycobacterial sonicates (Fig. 2) was dried, resuspended in hexane and chromatographed over a cyano (CN)-bonded silica solid-phase extraction (SPE) column (JT Baker). Four fractions were collected by eluting stepwise with hexane, 25% chloroform in hexane, 85% chloroform in hexane, and methanol. Before loading on a C18 column (Alltech Nucleosil C18 5 μm , 25 cm $\times$ 4.6 mm), the antigenic fraction (eluted with 85% chloroform in hexane from the CN-SPE column) was saponified with methanolic KOH (25% KOH in methanol, 85 °C 3–5 h, acidified with HCl, extracted with chloroform and neutralized) in order to cleave compounds covalently associated by ester linkages at the carboxyl end of the acyl chain, and finally derivitized with the ultraviolet



absorbing group *p*-bromophenacyl bromide (the phenacyl group attaches by an ester linkage on the free carboxylic acid end of an acyl chain)²¹. The large absorbance peak (A_{254}) seen eluting between 2 and 6 min (*a*, *c*, top panels) was free excess bromophenacyl bromide. A high *M*, standard (Std) was included in each run (Rib). Chromatograms had a general appearance similar to published results for mycolic acids²¹ and the broad peaks seen were due to the characteristic heterogeneity in acyl chain length of the mycolic acids. Because after derivitization antigenic activity was lost (unpublished data, E.M.B.), each fraction after HPLC was saponified again to remove the terminal phenacylbromide group, which restored antigenic activity. Fractions were resuspended in complete media/10% fetal calf serum and tested at a 1:17 dilution relative to the original sonicate volume. In *b*, trehalose containing compounds were saponified as for *a*. In *c*, 6,6'-trehalose dimycolate was prepared for HPLC analysis and assayed as for *a*. Saponified 6,6'-trehalose dimycolate (400 μg) was chromatographed and each fraction was tested at a final relative dilution of 1:10 in the proliferation assay.

FIG. 4 Specificity and CD1b restriction of the T-cell response to mycolic acid. **a**, T-cell lines specific for mycobacteria were tested for the ability to respond to total *M. tuberculosis* sonicates and the HPLC-purified mycolic acids from either mycobacterial sonicates or 6,6'-trehalose dimycolate. The responses of three representative mycobacteria-specific T-cell lines are shown: DN1 (top) (CD4⁺ CD8⁺, CD1b restricted, $\alpha\beta$ TCR⁺); DN6 (middle) (CD4⁺ CD8⁺, CD1c restricted, $\alpha\beta$ TCR⁺, (unpublished data, SP)); DG.1 (bottom) (CD4⁺, HLA-DR7 restricted, $\alpha\beta$ TCR⁺). APCs for all T-cell lines tested were identical GM-CSF plus IL-4-treated (CD1⁺) PBMC from an HLA-DR7⁺ individual. Two additional T-cell lines tested in the same experiment, SP-F3²⁹ (CD4⁺ $\alpha\beta$ TCR⁺, HLA-DR-restricted, tetanus toxoid specific) and CP.1.15³⁰ (CD4⁺ CD8⁺, $\gamma\delta$ TCR⁺, mycobacteria specific) also did not respond to purified mycolic acids (data not shown). **b**, Cytolytic response of the DN1 line to CD1 transfectants of C1R cells pulsed overnight with mycolic acid. CD1a, -b, -c or mock transfectants¹⁰ of C1R lymphoblastoid cells were pulsed with mycolic acids prepared from 6,6'-trehalose dimycolate or media alone and used as targets in a standard ⁵¹chromium release cytolytic assay. Cytolytic assays and calculation of per cent specific lysis were done as described^{10,12}. **METHODS.** In **a**, total *M. tuberculosis* (H37Ra, Difco) sonicates were titred over a range of 6 logs and the peak response is shown (dilution of 1:11,250 (DN1, DG.1) or 1:20,250 (DN6)) in the top panel. In the middle panel, four fractions (27–30) obtained from rphPLC purification (Fig. 3a) were pooled to be tested. The results shown are for an antigen dilution of 1:10,000, but identical results were found over a titration range of 3 logs. Bottom, three fractions (26–28) obtained from the HPLC (Fig. 3c) separation of 6,6'-trehalose dimycolate mycolic acids were pooled and tested in a final dilution of 1:12.5 (relative to amount of 6,6'-trehalose dimycolate loaded onto the HPLC column (Fig. 3c)). This corresponds to the amount of material purified from 200 μ l (one proliferation assay culture well) of a 12.5 μ g ml⁻¹ preparation of 6,6'-trehalose dimycolate. Identical responses were obtained over a 3 log titration. Background (media alone) c.p.m. were subtracted in all three panels. In **b**, data shown was obtained at an effector to target ratio of 50:1. Mycolic acid prepared from 6,6'-trehalose dimycolate by saponification (Fig. 3b) was diluted to a final concentration equivalent to 200 μ g ml⁻¹ of the starting 6,6'-trehalose dimycolate preparation (Sigma).



viewed as separate from MHC-encoded presentation. We propose that CD1 molecules present distinct antigens from mycobacteria and other microbial pathogens to specialized subsets of T cells. Presumably, CD1-restricted responses to lipids or hydrophobic antigens other than mycolic acid also may be

found. By including CD1 as a non-protein antigen-presenting molecule, the immune system appears to have extended the potential repertoire of foreign antigens recognized by $\alpha\beta$ T cells beyond the realm of those peptides that can bind to MHC class I, Ib and II molecules. □

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A potent peptidomimetic inhibitor of HSV ribonucleotide reductase with antiviral activity *in vivo*

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HERPES simplex viruses (HSV) types 1 and 2 encode their own ribonucleotide reductases (RNRs) (EC 1.17.4.1) to convert ribonucleoside diphosphates into the corresponding deoxyribonucleotides¹. Like other iron-dependent RNRs, the viral enzyme is formed by the reversible association of two distinct homodimeric subunits². The carboxy terminus of the RNR small subunit (R2) is critical for subunit association^{3,4} and synthetic peptides containing these amino-acid sequences selectively inhibit the viral enzyme by preventing subunit association⁴⁻⁹. Increasing evidence indicates that the HSV RNR is important for virulence and reactivation from latency¹⁰⁻¹⁴. Previously, we reported on the design of HSV RNR inhibitors with enhanced inhibitory potency *in vitro*^{4,15,16}. We now report on BILD 1263, which to our knowledge is the first HSV RNR subunit-association inhibitor with antiviral activity *in vivo*. This compound suppresses the replication of HSV-1, HSV-2 and acyclovir-resistant HSV strains in cell culture, and also strongly potentiates the antiviral activity of acyclovir. Most importantly, its anti-herpetic activity is shown in a murine ocular model of HSV-1-induced keratitis, providing an example of potent non-substrate-based antiviral agents that prevent protein-protein interactions. The unique antiviral properties of BILD 1263 may lead to the design of new strategies to treat herpesvirus infections in humans.

Structure activity studies were done using a competitive RNR binding assay⁴ to assess inhibitor potency. We based these studies on individually optimizing each of the six amino acids in the hexapeptide AVVNDL (single-letter code) corresponding to the carboxy terminus of the HSV R2. Figure 1 depicts the

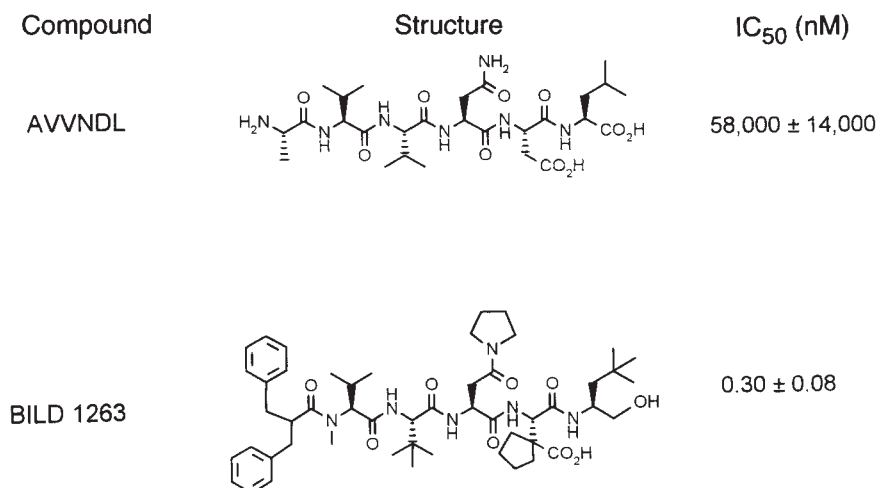
culmination of this investigation, BILD 1263. This inhibitor exhibits a ~200,000-fold improvement in potency over the peptide AVVNDL. More importantly, however, BILD 1263 also demonstrates antiviral activity *in vitro* and *in vivo*.

Dose-response curves for inhibition of HSV replication by BILD 1263 in cell culture are shown in Fig. 2a. Because RNR is not required for replication of HSV in exponentially growing cells¹⁰, experiments were done with serum-starved baby hamster kidney (BHK-21) cells. In a colorimetric viral yield assay¹⁷, the effective concentration of BILD 1263 required to inhibit HSV replication by 50 per cent (that is, EC₅₀) was 4 µM for HSV-2 (strain HG-52) and 3 µM for HSV-1 (strain F). The antiviral effect of BILD 1263 was not due to cytotoxicity because the antiviral activity was observed at concentrations considerably below the cytotoxic levels of the compound. The selectivity index in BHK-21 cells was ~30 with a 50 per cent cytotoxic concentration in mock infected cells (CC₅₀) of 100 µM as measured by means of a tetrazolium salt (MTT) metabolic assay¹⁸. A similar CC₅₀ value was obtained by using a cell proliferation assay to assess cytotoxicity (data not shown). Under our assay conditions acyclovir (ACV) inhibited growth of both viruses with an EC₅₀ of about 3 µM. The antiviral activity of BILD 1263 could also be demonstrated by measuring virus yield using a plaque assay¹⁹. In this assay, half-maximal reduction of 5 log₁₀ in progeny virus was achieved at 5 µM for BILD 1263 and 4 µM for ACV against HSV-2 (strain HG-52).

The antiviral activity of BILD 1263 extended also to ACV-resistant strains of HSV²⁰ (Fig. 2b). Two distinct mechanisms of ACV resistance have been defined at the molecular level. They involve mutations in either the viral thymidine kinase or DNA polymerase genes^{21,22}. For our study, we chose a collection of clinical²³ (615.8 and 615.9) and laboratory^{24,25} (PAA'5 and dlsptk) strains of HSV-1 viruses that represent both classes of ACV-resistant viruses. Whereas the degree of resistance to ACV ranged from 5.7- to 28-fold for the laboratory strains and from 21- to 71-fold for the clinical isolates (Fig. 2b, left), BILD 1263 appeared to exhibit similar potency against the ACV-resistant and parental wild-type strains (Fig. 2b, right). The sensitivity of all ACV-resistant HSV-1 strains to the RNR inhibitor is consistent with the molecular mechanism of ACV resistance. Collectively, these results highlight the important potential clinical utility of RNR inhibitors in the treatment of ACV-resistant HSV infections.

The effect of BILD 1263 on viral RNR-induced deoxyribonucleoside triphosphate (dNTP) pools in HSV-2-infected cells is shown in Fig. 3a. This inhibitor reduced the pools of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP) in HSV-2-

FIG. 1 Structure and potency of HSV RNR subunit-association inhibitors. The synthesis of BILD 1263 and a more in-depth discussion of the structure activity relationship will be published elsewhere. Compounds were tested in a competitive binding assay as described previously⁴. IC₅₀ (inhibitor concentration to give 50% inhibition) values represent the mean $\pm$ s.d. of 3 determinations. None of the compounds inhibited human RNR up to 500 µM when measured as described elsewhere⁷. Abbreviations for the amino-acid residues are: A, Ala; V, Val; N, Asn; D, Asp; L, Leu.



infected cells in a dose-dependent manner. The calculated IC_{50} s of BILD 1263 for inhibition of each dNTP pool were similar (dATP, 0.24 μ M; dCTP, 0.31 μ M and dGTP, 0.20 μ M). In mock infected cells, the inhibitor had no effect on the dNTP levels, even at the highest concentration tested, confirming that it does not affect the mammalian RNR (data not shown). Furthermore, no effect was observed on the level of the ribonucleoside triphosphate (rNTP) pools, suggesting that BILD 1263 did not affect cellular enzymes involved in rNTP biosynthesis. These results indicate that the antiviral activity of BILD 1263 is due to selective inhibition of the viral RNR in the infected cells.

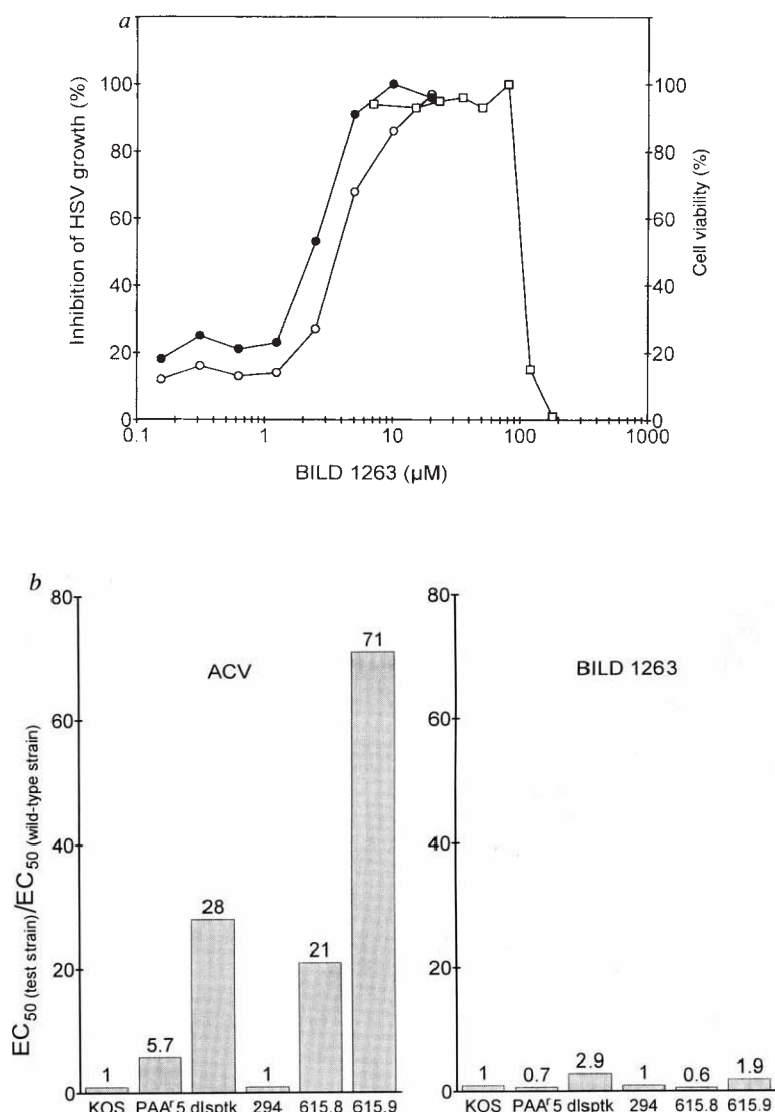
Selective HSV RNR inhibitors represent a new class of antiviral agents that may be potentially useful to improve existing therapies for HSV infections. In particular, it was of interest to determine whether these compounds could potentiate the antiviral effect of ACV because compounds that inhibit the formation of dGTP enhance the antiherpetic activity of ACV²¹, and HSV RNR null mutant viruses are reportedly hypersensitive to ACV²⁶. As shown by the isobole representation (Fig. 3b), BILD 1263 potentiates the ability of ACV to inhibit HSV-2 replication. For example, BILD 1263 at a concentration that produced 20% inhibition of HSV-2 replication caused the EC_{50}

of ACV to decrease about 20-fold. A similar degree of potentiation of ACV was also observed in confluent BHK-21 cells (data not shown). These results are consistent with the expected mode of action of RNR inhibitors and underline their potential clinical utility in combination therapy with ACV.

The combined antiviral properties of BILD 1263 in cell culture prompted us to investigate whether this inhibitor would be useful to treat HSV-induced disease. To determine whether BILD 1263 had antiviral activity *in vivo*, we used a murine ocular model²⁷ of HSV infection in which mice were infected with HSV-1 KOS and scored for the severity of stromal keratitis at various times postinfection. As shown in Fig. 4, BILD 1263 when administered as an ophthalmic cream prevented the development of stromal keratitis in a statistically significant manner at a concentration of both 1 and 5% (w/w). In comparison, ACV when given in the same formulation at a concentration of 1% (w/w), provided complete protection against HSV-induced keratitis (data not shown). A more detailed analysis of the *in vivo* study will be published elsewhere. These results demonstrate that BILD 1263 exhibits antiviral activity *in vivo* when applied topically and support the potential clinical utility of this class of inhibitors as antiviral agents to treat HSV infections in humans.

FIG. 2 Effect of BILD 1263 on HSV replication and cell viability in serum-starved cells. a, Wild-type HSV: HSV-2 strain HG-52 (○); HSV-1 strain F (●), cell viability (□). b, ACV-resistant HSV-1 strains.

METHODS. a, Stock solutions of compound were prepared by dissolving each compound in dimethylsulphoxide (DMSO), diluting with defined BBMT medium²⁸ (1% DMSO final concentration), sonicating for 20 min in ice-cold water and filtering through 0.22 μ m Millex (Millipore) filters. Serial dilutions of the stock solutions were added to infected cell monolayers prepared beforehand as follows. Baby hamster kidney (BHK)-21/C13 (ATCC CCL10) cells were seeded in 24-well plates in α -MEM (Gibco) medium containing 8% (v/v) fetal bovine serum (FBS) (Gibco) and incubated at 37 °C with 5% CO₂. After 6 h, the concentration of FBS was reduced to 0.5% (v/v) and the cells were serum-starved for 3 days. Serum-starved monolayers of BHK-21 cells were infected with either HSV-2 (strain HG-52) at a multiplicity of infection (MOI) of 0.02 plaque-forming units (PFU) per cell or HSV-1 (strains F and KOS) at a MOI of 0.01 PFU per cell in BBMT medium. After 1 h the HSV-infected cells were rinsed with BBMT medium and then incubated with the test compounds in BBMT medium. After 28 h of incubation at 37 °C, the infected cells were collected and the virus particles produced were quantified by a colorimetric (neutral red uptake) titration assay¹⁷ on BHK-21 cells or by a plaque assay on confluent Vero cells¹⁹. A typical dose-response curve is shown from the colorimetric assay. The EC_{50} s stated in the text are the means from 3 independent experiments. b, The EC_{50} s of ACV and of BILD 1263 against ACV-resistant HSV-1 strains were measured as described above. In all cases, serum-starved BHK-21 cells were infected at a MOI of 0.01 PFU per cell. The clinical isolate 294 is a wild-type HSV-1 strain. The clinical isolate 615.9 is a thymidine kinase negative mutant and 615.8 is a DNA polymerase mutant. The laboratory HSV-1 strains KOS, PAA'5 and dlsptk are wild type, DNA polymerase and thymidine kinase negative mutant HSV-1 strains, respectively. The results were normalized by dividing the EC_{50} of each test strain by the EC_{50} of the wild-type strain from which it is derived. This value is shown on top of each bar. In these experiments, the EC_{50} of ACV was 2.7 and 0.8 μ M against the wild-type HSV-1 strains KOS and 294, respectively. The EC_{50} of BILD 1263 against the same wild-type viruses was 2.2 and 2.5 μ M, respectively. The results are given as the average of triplicate measurements. Cytotoxicity of BILD 1263 for serum-starved BHK-21 cells was determined with a tetrazolium salt MTT assay¹⁸ under the same cell culture assay conditions used for the HSV replication assay.



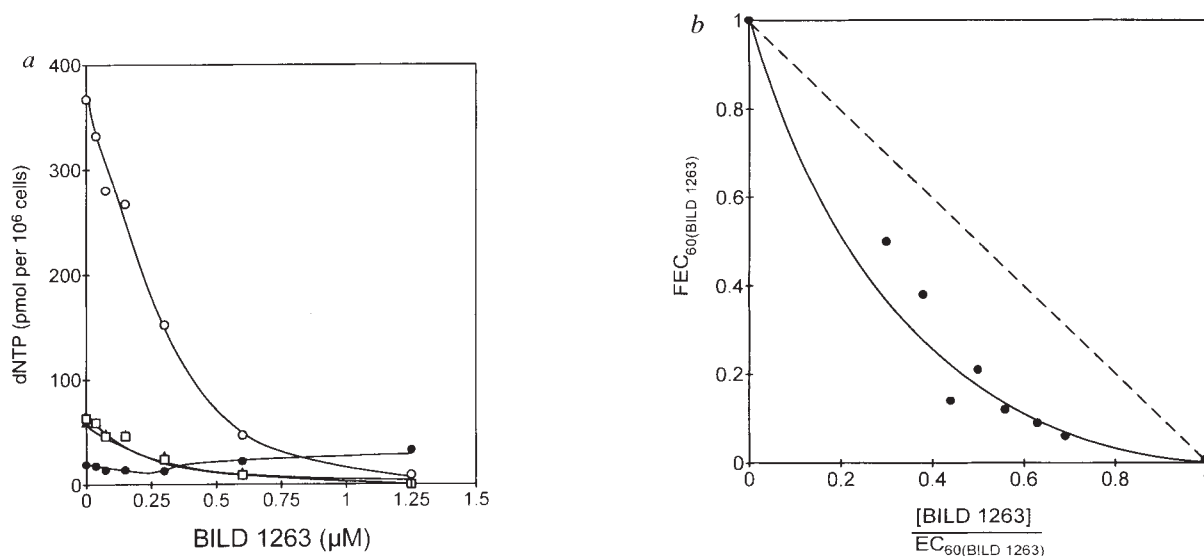


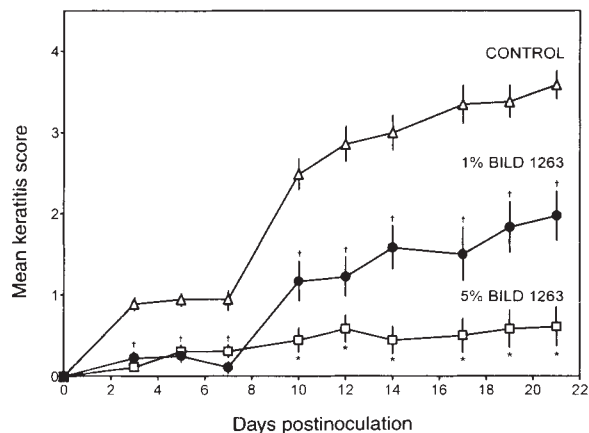
FIG. 3 Effect of BILD 1263 on HSV-2-induced dNTP pools in serum-starved cells (a) and synergy with ACV (b). dATP (○), dCTP (▲), dGTP (□) and dTTP (●).

METHODS. a, BHK-21 cells ($\sim 6 \times 10^6$ cells) in 100-mm Petri dishes, prepared as described in the legend to Fig. 2, were either mock infected or infected with HSV-2 strain HG-52 at a MOI of 10 PFU per cell. After a 1 h adsorption period at 37 °C in α -MEM (Gibco) medium containing 2% dialysed heat-inactivated FBS, the cells were incubated at 37 °C for 12 h with or without (control) increasing concentrations of BILD 1263. Then the cells were washed twice with ice-cold Hank's balanced salt solution. The nucleotides were extracted with ice-cold 60% methanol and the dNTPs were quantified by the periodate-methylamine method²⁹ with some modifications. After degradation of rNTPs, the samples were diluted to 1 ml total volume with double-distilled water (ddH₂O) and dialysed overnight against 4 l ddH₂O at 4 °C in Spectra/Por 3 dialysis bags. The dNTPs were quantified by strong anion exchange high-performance liquid chromatography (HPLC). The dialysis step was found useful to eliminate interfering peaks on the HPLC chromatograms. The data was used to calculate the percentage of

inhibition of dNTP formation as compared to untreated controls after subtraction of the dNTP pool levels in mock infected cells. The latter amounted to 5, 2, 1 and 10 pmol per 10⁶ cells for dATP, dCTP, dGTP and dTTP, respectively. At 12 h postinfection the dNTP pools in HSV-2-infected cells amounted to 367, 60, 63 and 19 pmol per 10⁶ cells for dATP, dCTP, dGTP and dTTP, respectively. IC₅₀ values for dNTP inhibition given in the text were derived from four independent experiments. b, Selected concentrations of BILD 1263 were tested in combination with various doses of ACV (Sigma) and the EC₅₀s in the HSV-2 replication assay were determined as described in the legend to Fig. 2. The isobole method was used to determine the type of interaction between the two compounds³⁰. The FEC_{60(ACV)} represents the ratio of the concentration of ACV required to inhibit viral replication by 60% (EC₆₀) in the presence of a fixed concentration of BILD 1263 to the concentration of ACV required in the absence of BILD 1263. The x-axis represents the ratio of the fixed concentration of BILD 1263 to the EC₆₀ of BILD 1263 in the absence of ACV. The theoretical plot for non-interacting inhibitors is indicated by the dashed line. In this method, displacement of the data points to the left is indicative of synergistic behaviour.

FIG. 4 Effect of topical BILD 1263 on HSV-1-induced ocular disease in mice. Control (Δ), ophthalmic formulation only; 1% BILD 1263 (●); 5% BILD 1263 (□).

METHODS. Female BALB/C mice (3–4 weeks old) were used in the studies. Following halothane anaesthesia, the cornea was scratched 3 times vertically and 3 times horizontally with a sterile 30-gauge needle. Alpha-MEM (5 μl) containing 1×10^6 PFU of HSV-1 strain KOS was placed on the cornea and left in place for 30 s. Excess virus was removed with a cotton swab. The severity of ocular disease (keratitis) was scored at various times postinoculation for 21 days. Keratitis was scored as: 1+, cloudiness of the cornea, some iris detail visible; 2+, iris detail obscured; 3+, cornea totally opaque; and 4+, cornea ulcerated or perforated. The results were reported as mean disease score for each group of 12 mice on each observation day. The data shown was derived from 3 independent experiments. Mice were treated 6 times per day with an ophthalmic formulation for the first three days, starting 3 h after inoculation and subsequently 4 times per day for the next 4 days. The ophthalmic formulation consisted of 15% H₂O, 15% white mineral oil (paraffin oil), 70% wool alcohol ointment (Eucerin anhydrous, Smith & Nephew). BILD 1263 was ground in the ointment to a final concentration of 5 and 1% (w/w). Statistics were done by the analysis of variance (ANOVA) using SAS software (Cary, NC). Differences between groups were tested by using Student–Newman–Keuls multiple comparison. A value of $P < 0.05$ was considered to be statistically significant. *Statistically different from control group and 1% treatment group; †statistically different from control group.



In conclusion, we have designed a new class of selective HSV RNR subunit-association inhibitors with subnanomolar potencies. The inhibitor BILD 1263 described here is the most potent HSV RNR inhibitor reported to date. In addition to increased inhibitory potency against HSV RNR, this compound also inhibits the replication of HSV-1, HSV-2 and ACV-resistant mutant strains of HSV-1 in cell culture and strongly potentiates the antiviral activity of ACV. Most importantly, BILD 1263 can reduce the severity of HSV-1-induced disease in a murine ocular model. Our results therefore indicate that HSV RNR subunit-association inhibitors alone or in combination with ACV may be clinically useful therapeutic agents for the treatment of HSV infections due to ACV-sensitive or ACV-resistant strains.

To our knowledge, BILD 1263 is the first peptidomimetic inhibitor with antiviral activity *in vivo* that acts by disrupting an essential protein-protein interaction between two enzyme subunits. Thus, our results support the concept of disruption of protein-protein interactions as a general strategy of inhibitor design for the development of new chemotherapeutic agents. □

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Ypt1p implicated in v-SNARE activation

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SYNAPTOBREVIN-LIKE membrane proteins that reside on transport vesicles, called the vesicle SNARE (v-SNARE), play a key role in ensuring that a vesicle targets and fuses with its correct acceptor compartment^{1–3}. Here we show that Bos1p, the v-SNARE of yeast endoplasmic reticulum-to-Golgi transport vesicles, pairs with another integral membrane protein of similar topology (Sec22p) on vesicles. This pairing, which appears to require functional Ypt1p (Rab in mammalian cells), may aid the activity of Bos1p on this compartment. These findings suggest that Rabs regulate the specificity of membrane fusion by selectively activating the v-SNARE on carrier vesicles. Because the v-SNARE resides on more than one membrane, such a regulated activation step may be necessary to prevent the premature fusion of donor and acceptor compartments⁴.

Ypt1p colocalizes with Bos1p and Sec22p on vesicles⁵. Genetic studies suggest a possible physical interaction among these proteins^{6–8}. To address this point, coprecipitation experiments were done with detergent solubilized endoplasmic reticulum (ER)-to-Golgi transport vesicles that were formed *in vitro*. When affinity-purified antibody (α -Bos1p), that specifically crossreacts with Bos1p (Fig. 1a, lanes 1 and 2), was used to precipitate detergent-solubilized vesicles (Triton X-100 or CHAPS), a band of M_r 25 K coprecipitated with Bos1p (Fig. 1b, lanes 2 and 5). This band was precipitated by antibodies against Sec22p (α -Sec22p; lane 3), but not α -Ypt1p (lane 4), demonstrating that it is Sec22p. Crosslinking studies were used to assess these interactions by a second method. A 25 K species was crosslinked to Bos1p with dithio-bis-(succinimidylpropionate) (DSP) (Fig. 1c,

lane 4). This band, which was recognized by α -Sec22p (Fig. 1d, compare lanes 1 and 2), was not observed when the crosslinker was inactivated (Fig. 1b, lane 1). Sec22p could be crosslinked to Bos1p in the absence (Fig. 1c, lane 4) or presence of Triton X-100 (lane 3), but not when the sample was denatured (Fig. 1c, lane 2 and Fig. 1d, lane 1). When the non-reduced complex was analysed on a 12% SDS-polyacrylamide gel, it remained as a high M_r mass at the top of the gel (not shown), implying that Bos1p forms a heterooligomeric complex with Sec22p.

Bos1p only appears to interact with Sec22p on vesicles, the site of Bos1p function⁵. Although these proteins are present in the permeabilized yeast cell preparations (PYCs) used to form vesicles *in vitro*⁵, significant amounts of Sec22p could not be crosslinked to Bos1p in the presence (Fig. 1c, lane 1) or absence (not shown) of detergent. Furthermore, most of the Sec22p that resides within the PYCs failed to coprecipitate with Bos1p (compare lanes 1, 2 and 3 in Fig. 2a with lanes 1 and 2 in Fig. 2b) from Triton X-100 solubilized cells.

A prediction of these findings is that the Bos1p-Sec22p complex should accumulate in a class of temperature-sensitive (ts) secretory mutants that build-up ER-to-Golgi transport vesicles (*sec17* and *sec18*) at 37 °C^{9,10}. To test this hypothesis, detergent-solubilized extracts of *sec17* and *sec18* prepared from spheroplasts that were regenerated at 37 °C, were treated with α -Bos1p. Wild-type and *sec12*, a mutant that fails to bud vesicles from the ER^{9,10}, were also examined. A small amount of the complex was precipitated from wild-type extracts (Fig. 3a, lane 1), reflecting the fact that vesicles en route to the Golgi are present in these cells. In contrast, significantly larger amounts of Sec22p coprecipitated with Bos1p from *sec18-1* (compare lanes 1 and 2) and *sec17-1* (compare lanes 1 and 4) extracts, but not from *sec12-4* (lane 3).

The relationship between *BOS1* and *SEC22* was explored further by examining their genetic interactions in greater detail. Previous studies have shown that although *BOS1* is an essential gene, *SEC22* is not^{7,11}. Strains disrupted for *SEC22* (SFNY361) grow well at 30 °C⁷ (Fig. 4a, compare a and d), but are temperature sensitive and cold-sensitive for growth⁷. The overproduction of *BOS1* dramatically restored the growth of SFNY361 at 34 °C (Fig. 4a, compare c and d with a), whereas an additional copy

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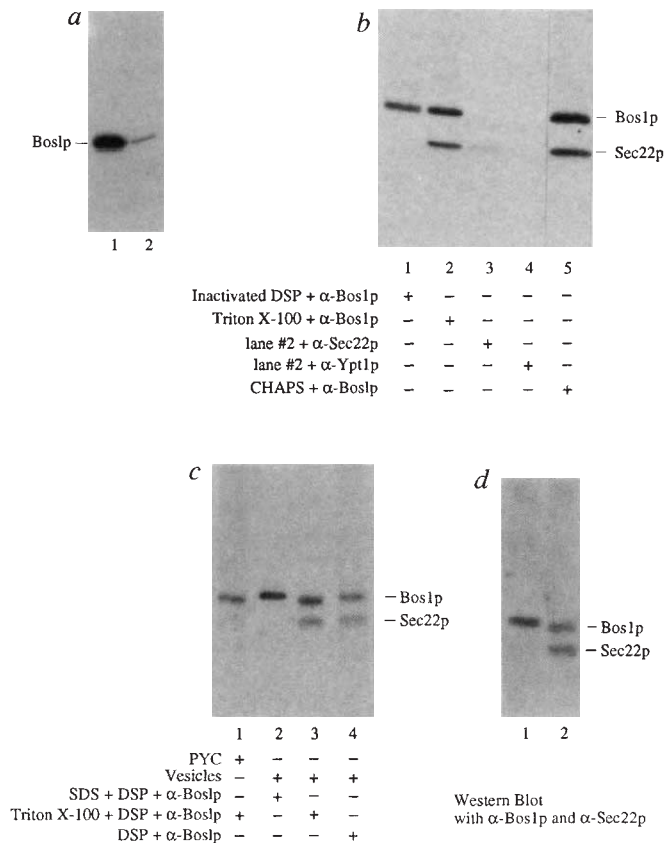


FIG. 1 Bos1p physically interacts with Sec22p on vesicles. **a**, Lane 1, western blot of lysate overproducing BOS1 or normal levels of BOS1 (lane 2). **b**, Lane 1, vesicles ($5-8 \times 10^6$ c.p.m.) treated with inactivated DSP (2 M Tris, pH 8.0) were solubilized with SDS before treatment with α -Bos1p; lane 2, vesicles solubilized with 1% Triton X-100 and precipitated with α -Bos1p. The precipitation of Sec22p by α -Bos1p was competed with excess recombinant Bos1p; lane 3, sample in lane 2 was re-precipitated with α -Sec22p; lane 4, sample in lane 2 was re-precipitated with α -Ypt1p; lane 5, vesicles solubilized with 2% CHAPS and precipitated with α -Bos1p. Most, if not all, the Sec22p on vesicles is associated with Bos1p. **c**, Lane 1, PYCs (6 optical density units at 599 nm) crosslinked with DSP in the presence of 1% Triton X-100; lane 2, vesicles solubilized with 1% SDS and treated with DSP; lane 3, vesicles solubilized with 1% Triton X-100 and treated with DSP; lane 4, vesicles treated with DSP in the absence of detergent. **d**, Lanes 1 and 2, western blot using α -Bos1p and α -Sec22p antibodies sequentially on samples in **c**, lanes 2 and 3.

METHODS. The PYCs used to form radiolabelled vesicles (20 reactions) were incubated with 5mCi of 35 S-trans label for 6 h at 30 °C. Crude vesicles⁵ were solubilized with PBS, 1% Triton X-100 (or 2% CHAPS) and 250 mM sorbitol before the soluble material was treated with 30 μ g purified α -Bos1p (1–2 h, 4 °C). For crosslinking studies, crude vesicles (100 μ l) ($\pm 1\%$ Triton X-100) were incubated with 2% (v/v) DSP (30 min, 25 °C). The DSP was added from a 10 mg ml⁻¹ stock that was prepared in dimethyl sulphoxide. The reactions were terminated with 1 vol 0.2 M ammonium acetate (10 min, 4 °C) and the samples were adjusted to 1% SDS, heated to 85 °C (5 min) and diluted (PBS, 0.5% Triton X-100, 0.1% BSA). The soluble material was incubated with α -Bos1p. The PYCs ($\pm 1\%$ Triton X-100) were treated with 3% (v/v) DSP (30 min, 25 °C), heated (65 °C, 10 min) and diluted (0.1% SDS, 1% Triton X-100) before precipitation with α -Bos1p. Samples were solubilized in the presence of 100 mM dithiothreitol (25 °C, 20 min) and then heated to 100 °C for 5 min.

of BOS1 (*CEN*) weakly suppressed the temperature sensitive growth defect (Fig. 4a, compare *b* and *d*). The overproduction of SEC22 could not substitute for the loss of BOS1 (not shown). Thus, these data indicate that BOS1 and SEC22 are not functionally equivalent. Rather, Sec22p may act by increasing the efficacy of Bos1p.

To address the possibility that Ypt1p regulates the formation of the Bos1p–Sec22p complex, we examined the *ypt1^{ts}* mutant that accumulates vesicles at 37 °C¹². Previous studies have shown these vesicles contain Bos1p and Sec22p¹³. Although the *ypt1^{ts}* mutant synthesizes normal levels of Sec22p (Fig. 3c, compare lanes 1, 2 and 3), significant amounts of Sec22p failed to coprecipitate with Bos1p (compare *ypt1^{ts}* in Fig. 3b, lane 3 with *sec18-1* in lane 2). As *ypt1^{ts}* is not completely restrictive for growth at 37 °C, a small amount of the complex ($\sim 0.6 \times$ wild type) was observed (compare lanes 3 and 1 in overexposed autoradiogram in Fig. 3b). More Sec22p was precipitated from *ypt1^{ts}* extracts when the spheroplasts were regenerated at 25 °C (Fig. 3b, compare lanes 3 and 4). This may reflect the fact that the complex forms at 25 °C on vesicles en route to the Golgi. Similar results were also obtained when a second mutant allele (*ypt1-3*) was examined.

If Ypt1p stimulates the interaction of Bos1p with Sec22p, the co-overexpression of BOS1 with SEC22 should bypass the need for Ypt1p and restore the complex on vesicles. To test this hypothesis, we transformed a diploid strain (NY921) disrupted for one copy of *YPT1* (see legend to Fig. 4B) with plasmids that overexpress BOS1 (pPL101) and SEC22 (pJG103) and then did tetrad analysis. For comparison, we also transformed NY921 with either pPL101 or pJG103. BOS1 alone could not compensate for the loss of *YPT1* (not shown), and the suppression by SEC22 was extremely weak (that is, the microcolony in 7B II, Fig. 4B was not apparent). This latter finding was reported previously⁷. In contrast, the loss of *YPT1* was efficiently bypassed when SEC22 was co-overexpressed with BOS1 (see

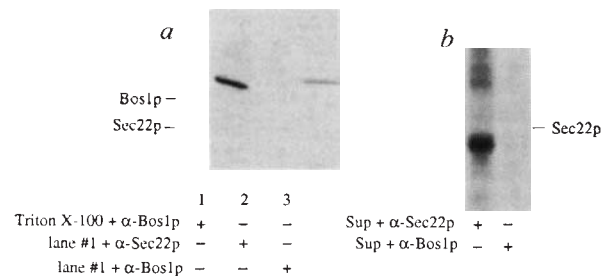


FIG. 2 Anti-Bos1p antibody does not precipitate Sec22p from detergent-solubilized PYCs. **a**, Lane 1, detergent-solubilized PYCs precipitated with purified α -Bos1p; lane 2, sample in lane 1 re-precipitated with α -Sec22p; lane 3, sample in lane 1 re-precipitated with α -Bos1p. **b**, The supernatant from the precipitate shown in **a**, lane 1 was re-precipitated with α -Sec22p (lane 1) and α -Bos1p (lane 2).

METHODS. Fractions used in the coprecipitation experiments were made from cells that were radiolabelled with 35 S-methionine as in Fig. 1. PYCs (6 A₅₉₉ units), prepared as described before^{18–20}, were solubilized with PBS containing 2% Triton X-100, 250 mM sorbitol and 0.1% BSA. Detergent solubilized PYCs were centrifuged (5 min, 4 °C) in a microfuge and the resulting supernatant was incubated with antibody. The protein complexes were precipitated with protein A Sepharose (90 min, 4 °C), washed as described below, and solubilized in sample buffer. Half the sample was analysed by SDS–polyacrylamide gel electrophoresis, while the remaining portion was diluted with PBS containing 1% Triton X-100 with 0.1% BSA and re-precipitated with α -Sec22p or α -Bos1p. The beads were washed twice with IP buffer (PBS, 0.1% SDS, 0.5% Triton X-100), transferred to a new tube, washed 6 times with IP buffer, once with 20 mM Tris (pH 8.0) and solubilized with sample buffer. The entire sample was analysed on a 15% SDS–polyacrylamide gel.

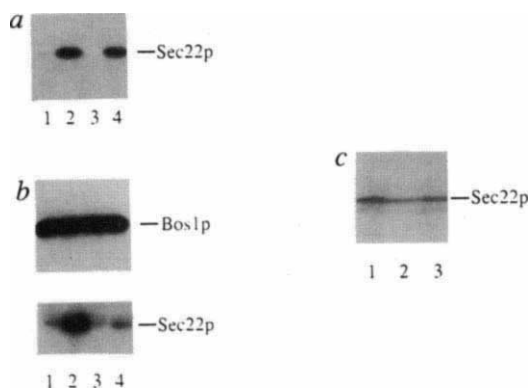


FIG. 3 The Bos1p-Sec22p complex does not form in the absence of functional Ypt1p. a, Lane 1, SFNY26-6A (*MATa*, *his4-619*) at 37 °C; lane 2, NY432 (*MATa*, *sec18-1*, *ura3-52*) at 37 °C; lane 3, NY738 (*MATa*, *sec12-4*, *ura3-52*) at 37 °C; lane 4, NY418 (*MATa*, *sec17-1*, *ura3-52*) at 37 °C. b, Lane 1, SFNY26-6A at 37 °C; lane 2, NY432 at 37 °C; lane 3, SFNY330 (*MATa*, *ypt1^{ts}-LEU2*, *leu2*, *his3*) at 37 °C; lane 4, SFNY 330 at 25 °C. c, About 0.5 mg of lysate was precipitated with TCA as described before¹⁴. The precipitate was solubilized in sample buffer and electrophoresed. Lane 1, SFNY26-6A at 37 °C; lane 2, NY432 at 37 °C; lane 3, SFNY330 at 37 °C. The autoradiograms were exposed for 18 h (a), 67 h (b) and 66 h (c).

METHODS. Cells were grown in YPD medium to early log phase and converted to spheroplasts during a 60–90 min incubation at 25 °C using protocols described before^{18–20}. The spheroplasts were regenerated during a 1 h incubation at 37 °C or 25 °C and lysed as described elsewhere²¹. The lysate was centrifuged at 120,000g for 1 h and the supernatant was frozen in liquid nitrogen. Aliquots (2 mg of protein) were diluted to 1 ml (0.5% Triton X-100 final concentration) and incubated for 120–150 min with α -Bos1p before the protein complexes were precipitated with protein A Sepharose. The samples were washed twice with lysis buffer (containing 0.5% instead of 2% Triton X-100), transferred to a new tube, washed 6 times with the same buffer, once with 20 mM Tris (pH 7.0) and solubilized in 40–50 μ l of sample buffer. These precipitates were electrophoresed and blotted with α -Sec22p or α -Bos1p.

colonies 1C, 2A and 8C in I). Because high-copy plasmids are lost at a high frequency, the disrupted colonies that contain both plasmids (*His*⁺, *Leu*⁺ and *Ura*⁺) were of variable size (compare colony 1C with 3A and 3C in I). The Bos1p-Sec22p complex was precipitated from an extract prepared from one of the larger colonies (Fig. 4C, compare the amount of Sec22p in lane 2 with a wild-type strain that lacks the plasmids in lane 1).

Previous *in vitro* studies have shown that Bos1p specifically functions on vesicles⁵. Our findings suggest that Sec22p aids the activity of Bos1p on vesicles, allowing this v-SNARE to interact more efficiently with its receptor or t-SNARE (proposed to be SED5)¹⁴. In the absence of functional Ypt1p, the Bos1p-Sec22p complex fails to form. Because Ypt1p does not directly interact with Bos1p and Sec22p, the most likely interpretation of our data is that Ypt1p initiates a cascade of events that ultimately leads to the formation of the Bos1p-Sec22p complex. Furthermore, as *YPT1* encodes an essential gene and *SEC22* does not⁷, the *YPT1* gene product cannot act solely through *SEC22* (see below). We propose that Rabs regulate the specificity of vesicular transport by promoting the oligomerization of the v-SNARE on transport vesicles. The results presented here could explain why the yeast SNARE complex fails to form in *ypt1* mutant extracts¹⁵.

BET1, which also interacts with this group of genes^{6,7}, encodes a type II integral membrane protein^{7,16}. However, although Bos1p and Sec22p are always found on vesicles, Bet1p is not^{5,13}. Bet1p forms a complex with Bos1p on the ER, before the interaction of Bos1p with Sec22p (J.P.L. and S.F.N., unpublished observations). Interestingly, like *SEC22*, the overexpression of *BET1* also inefficiently compensates for the loss of Ypt1p⁷. Perhaps Ypt1p stimulates the formation of the Bos1p-Sec22p complex through Bet1p. For example, Bet1p may partially activate or prime Bos1p to interact with Sec22p as vesicles bud from the ER. *SEC21*, the fifth member of this group of interacting genes^{6,7}, encodes the homologue of a subunit of mammalian coatomer¹⁷. Coatomer may sequester the active v-SNARE (Bos1p-Sec22p) until fusion occurs. Further experiments will be

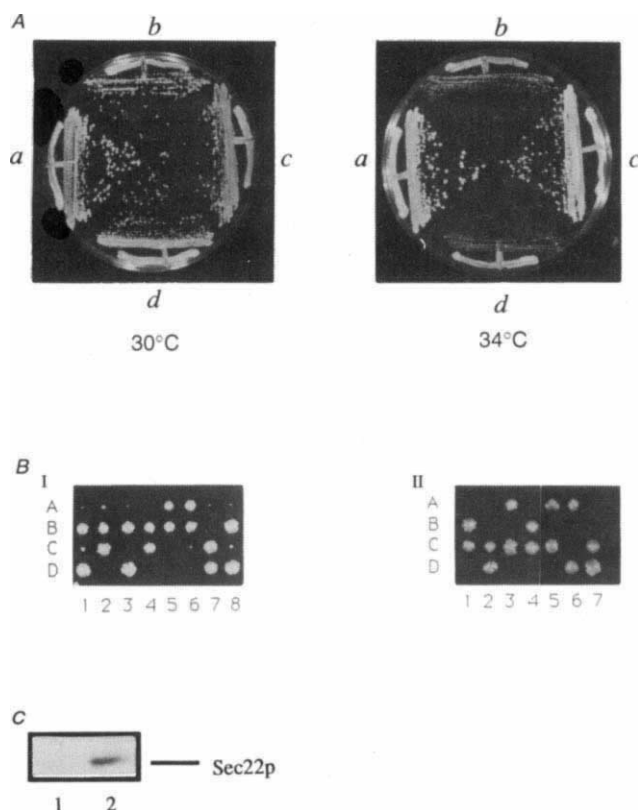


FIG. 4 The loss of Ypt1p is efficiently bypassed when Sec22p is co-overexpressed with Bos1p, a protein that substitutes for the loss of Sec22p. A, a, Wild type; b, SFNY361, pSFNB248 (*BOS1*, *CEN*); c, SFNY 361, pPL101 (*BOS1*, 2 μ m); d, SFNY361 at 30 °C and 34 °C. B, in NY921 (*MATa/a*, *ura3-52/ura3-52*, *leu2-3*, *112/leu2-3*, *112*, *his3- Δ 200/his3- Δ 200*, *ypt1 Δ ::HIS3*) was transformed with pPL101 (*BOS1*, *LEU2*, 2 μ m) and pJG103 (*SEC22*, *URA3*, 2 μ m), sporulated and subjected to tetrad analysis. The tetrads were photographed after six days as in II. Of the 23 tetrads that were analysed, 22 colonies were *His*⁺, *Leu*⁺ and *Ura*⁺. The larger colonies secreted significant amounts of invertase. In II, NY921 was transformed with pJG103, sporulated and subjected to tetrad analysis. c, SFNY26-6A at 25 °C; lane 2, SFNY456 (*his3- Δ 200*, *ypt1 Δ ::HIS3*, *ura3-52*, pJG103 (*SEC22*, *URA3*, 2 μ m), *leu2-3*, *112*, pPL101 (*BOS1*, *LEU2*, 2 μ m) at 25 °C. Extracts were prepared and precipitated with α -Bos1p as described in the legend to Fig. 3.

METHODS. SFNY361 (*MATa*, *GAL*⁺, *leu2-3*, *112*, *ura3-52*, *sec22 Δ ::URA3*) was transformed with either pPL101 (*BOS1*, *LEU2*, 2 μ m) or pSFNB248 (*BOS1*, *LEU2*, *CEN*) and suppression was tested at a variety of temperatures. Plasmid pPL101 efficiently suppressed the growth defect of SFNY 361 at 34 °C and restored the secretion of invertase. This strain was constructed by transforming SFNY 259 (*MATa/a*, *GAL*⁺/*GAL*⁺, *ura3-52/ura3-52*, *leu2-3*, *112/leu2-2*, *112*) with a linear *Bam*H-XhoI fragment that contains the *SEC22* gene disrupted with *URA3*. This disruption was constructed by inserting a *Ssp*I-*Bam*HI fragment, containing *URA3*, into the *Hinc*II, *Bcl*I sites of pJP1. This *Hinc*II, *Bcl*I digest removes the entire N-terminal cytoplasmic domain of *SEC22*. The *Ura*⁺ transformants were purified and tetrad analysis was done. SFNY361 was a product of this analysis. This strain is ts (34 °C–37 °C) and cs (15 °C) for growth. Suppression by *BOS1* was observed over a wide temperature range (15 °C–37 °C).

needed to determine the precise mechanism by which these events take place. □

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A highly conserved eukaryotic protein family possessing properties of polypeptide chain release factor

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THE termination of protein synthesis in ribosomes is governed by termination (stop) codons in messenger RNAs and by polypeptide chain release factors (RFs). Although the primary structure of prokaryotic RFs and yeast mitochondrial RF is established^{1–4}, that of the only known eukaryotic RF (eRF)⁵ remains obscure. Here we report the assignment of a family of tightly related proteins (designated eRF1) from lower and higher eukaryotes which are structurally and functionally similar to rabbit eRF. Two of these proteins, one from human⁶ and the other from *Xenopus laevis*⁷, have been expressed in yeast and *Escherichia coli*, respec-

TABLE 1 Polypeptide chain release activity of purified *X. laevis* C11 (expressed in *E. coli*) and human TB3-1 (expressed in yeast) proteins

Protein	Tetranucleotide added	Antibodies added	f[³⁵ S]Met released (pmol)
His-tagged C11	UAAA	—	0.38
	UGAA	—	0.39
	UAGA	—	0.35
	poly (A, G, U)	—	0.28
	UGGA	—	0
	UAAA	Anti-C11	0
His-tagged human protyrosine	UAAA	Anti-Eg5	0.35
	UAAA	—	0
His-tagged HIV reverse transcriptase	UAAA	—	0
	UAAA	—	0.20
	UGAA	—	0.23
	UAGA	—	0.17
TB3-1	UGGA	—	0

The RF activity was measured *in vitro* as stop-codon-dependent hydrolysis of f[³⁵S]Met-tRNA^{met} associated with AUG–80S-ribosome complex and a tetranucleotide that either did or did not encompass one of the three stop codons, or poly(A,G,U) as described^{15,19,23}. The incubation mixture (25 µl) contained 20 mM Tris-HCl pH 7.5, 15 mM MgCl₂, 8 mM NH₄Cl, 2 mM KCl, 0.1 mM GTP, 1.5 pmol f[³⁵S]Met-tRNA^{met}-AUG-ribosome complex, 0.1 A₂₆₀ of poly(A,G,U) or 0.05 A₂₆₀ of one of the tetranucleotides as indicated, and 0.5 µg of purified protein as indicated. The purification of the C11 and TB3-1 proteins was as described in Fig. 3 legend, whereas the other two proteins were purified by affinity chromatography on Ni-NTA resin (QIAGEN). Quantification of f[³⁵S]Met release was as described¹³. The activity was calculated as the amount of f[³⁵S]Met released in the presence of oligonucleotides; the value (0.05–0.1 pmol) of f[³⁵S]Met released in the absence of tetranucleotide has been subtracted from all values. Polyclonal anti-C11 antibodies were obtained as described⁷. Polyclonal antibodies directed against Eg5 (ref. 24; a kinesin-related protein involved in the mitotic-spindle formation) were used in a control experiment. Anti-C11 and anti-Eg5 antibodies were affinity-purified as described⁷. Where indicated, *X. laevis* C11 protein (0.5 µg) was incubated with 5 µl of anti-C11 or anti-Eg5 antibodies at 4 °C for 30 min and then added to the incubation mixture.

tively, purified and shown to be active in the *in vitro* RF assay. The other protein of this family, sup45 (sup1) of *Saccharomyces cerevisiae*, is involved in omnipotent suppression during translation^{8–12}. The amino-acid sequence of the eRF1 family is highly conserved. We conclude that the eRF1 proteins are directly implicated in the termination of translation in eukaryotes.

Rabbit eRF has been identified and partially purified^{5,13–15}. Eukaryotic RF complementary DNA has been cloned and sequenced¹⁶. The deduced eRF amino-acid sequence has nearly 90% homology with mammalian tryptophanyl-transfer RNA synthetases (TrpRS)^{17,18}, and therefore it was suggested¹⁸ and has been proved¹⁹ that 'eRF' is in fact TrpRS. As the main source of misassignment of 'eRF' was traces of TrpRS in the eRF preparation¹⁹, our first goal was to purify eRF to homogeneity. After the last purification step, the protein fractions showing the highest eRF activity (Fig. 1a) yielded a predominant band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1b) which was slightly lower (*M_r* ~ 50K) than those of partly purified rabbit eRF (54K)^{5,15} and bovine TrpRS (55K)¹⁷. The 50K protein band was extracted from the gels and subjected to two-dimensional gel electrophoresis followed by microsequencing²⁰.

Four tryptic peptides derived from rabbit eRF were sequenced (Fig. 2) and seemed to be identical or very similar to those present in human TB3-1 (ref. 6), *X. laevis* C11 (ref. 7), *S. cerevisiae* sup45 (sup1)¹⁰ and the *Arabidopsis thaliana* sup45-like proteins. All these proteins were structurally very similar (Fig. 2), with the exception of the carboxy terminus of the predicted

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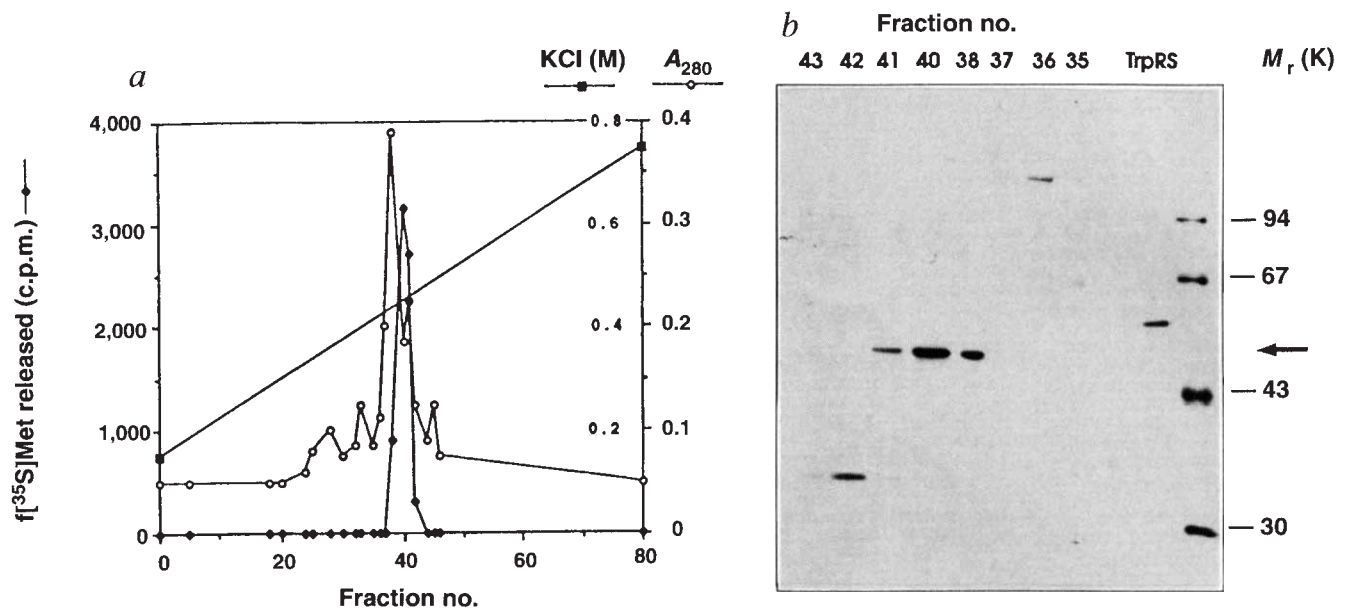


FIG. 1 Mono-Q column chromatography of rabbit eRF prepurified on Q-Sepharose and Bio-Rex columns. **a**, Proteins (2 A_{280} units) were applied to a Mono-Q column HR 5/5 using FPLC (Pharmacia) and equilibrated with buffer A (10 mM Tris-HCl pH 7.8, 1 mM dithiothreitol (DTT) and 0.1 mM EDTA) containing 0.15 M KCl; the column was washed with 10 ml of the same solution and proteins were eluted with 20 ml of a linear 0.15–0.75 M KCl gradient in buffer. **a**, Samples (10 μl) from the gradient fractions were used to measure the RF activity in the presence of poly(A,G,U) as described for Table 1. **b**, SDS-PAGE (12.5% acrylamide) of Mono-Q protein fractions (15 μl) after Coomassie blue staining. M, Protein markers; TrpRS, bovine tryptophanyl-tRNA

synthetase. Arrow indicates the position of eRF.

METHODS. The first steps of purification (ammonium sulphate precipitation of the 100,000g supernatant from rabbit reticulocyte lysate and chromatography on Q-Sepharose (Fast Flow) were as described¹⁹. The combined fractions (100 A_{280} units) containing RF activity after Q-Sepharose column were dialysed against buffer A and applied on Bio-Rex (Bio-Rad) column (1.6 $\times$ 8 cm) equilibrated with the same buffer followed by elution with 60 ml of a linear 0–0.5 M KCl gradient in buffer A. Fractions showing RF activity eluted between 75 and 200 mM KCl were pooled and loaded onto the Mono-Q column.

TB3-1 protein⁶ possibly resulting from errors in its cDNA nucleotide sequence. This was verified by resequencing the TB3-1 cDNA clone⁶ (not shown) and by sequencing a cDNA isolated from a human cDNA library using the *X. laevis* C11 DNA as a probe⁷; the amino-acid sequences deduced from the human C11 and the resequenced TB3-1 cDNA clones were identical and

possessed 98% homology with the *X. laevis* C11 protein (Fig. 2).

In addition, the four peptides derived from the rabbit eRF were completely identical to or differed insignificantly from the respective human and *X. laevis* C11. This observation implied that the primary structure of rabbit eRF is probably identical or nearly identical to the respective vertebrate (human and frog) protein sequences, making it unnecessary to isolate and sequence the rabbit eRF cDNA clone. The difference between vertebrate peptides (peptides c and d; Fig. 2) and the respective lower-eukaryotic peptides (Fig. 2) is probably due to the vast evolutionary distance between these species. Nevertheless, yeast sup45 shows unusually high similarity to vertebrate sequences⁷.

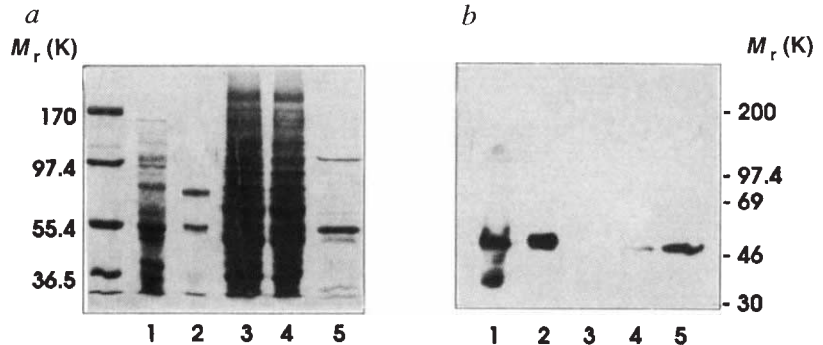
The similarity of these proteins was confirmed by immunochemical data: polyclonal antibodies raised against *X. laevis* C11 efficiently crossreacted with yeast-expressed human TB3-1 (Fig.

MADDPSSAADRNVIEWIKKLIKLSLEAARGNGTSMISLIIPKQDSRVAK	50	HuTB3/C11
*****	50	X1C11
*DNE---VEK*I***V***VQ***K*****V***GL*PLYQ*	47	YSUP45
QE*---K*IG***T*****M**R**VA**T*	48	ArabSUP45
(a) LSVLGAITSVQOR		
MLADEFGTASNIKSRVNRISVLGAITSVQORIKLVNKPNGLVVYCGTI	100	HuTB3/C11
*****	100	X1C11
VS*****S*****K*****TL******L**D*	97	YSUP45
VS*****S*****A*****L*****T**D*	98	ArabSUP45
VTEGKREKKNVIDPEPPKPIINTSLYLCDNKFHTRALTALLSDSKFPIV	150	HuTB3/C11
*****	150	X1C11
I**D*****FF**I***V*****V*SR**QA*D*****	147	YSUP45
DD***T*****A*****P*NE**ES*D*****	148	ArabSUP45
(b) FTVDLIP		
IDSGALFGTLCQNTREVILHKFTVDLPKKHGRGGQSALRPARLRMEKRHN	200	HuTB3/C11
*****	200	X1C11
M**Q*T***SVS*****T*****E*****E*****	197	YSUP45
M**N**T***S*****S*****S*****S*****S*****	198	ArabSUP45
YVRKVAETAVQLFIS--GDKVNVAGLVLAGSADFTKELSSQDMFQRLQS	248	HuTB3/C11
*****	248	X1C11
*****V***N**T--N*****K*****D*AK*EL**P*AC	245	YSUP45
*****T**L*TFY*NPATSQP**S*****E*****EL**P**A	248	ArabSUP45
KVLKLVDSISYGGENGFPQAIELSTEVLSNVKFIQKKLIGRYFDEISQDT	298	HuTB3/C11
*****	298	X1C11
ISIV*****A**A**A**YV*****LEA*****E*****	295	YSUP45
*I*NV**V*****T*****A**I*****K*****E*****	298	ArabSUP45
(c) YVLHCQGTERR ILVLT		
GKYCFGVEDTLKALEMGAVEILIVYENLDIMRYVLHCQGTERRKILYLT	348	HuTB3/C11
*****	348	X1C11
V*ID**DL***K***P***RTI**TPKDAEDN*-V*KFA*	344	YSUP45
VL*TFY*NPATSQP**S*****E*****EL**P**A	348	ArabSUP45
EOEK(d)		
EQEKDSHTDKETGQREHLIESMPLLEWPNANNYKFGATLEIVTDKSR	398	HuTB3/C11
*****	398	X1C11
*A*****FAI**A*****MDVVSE**I**L**A**N*****PI*****S	393	YSUP45
D**NNQ*N**H**A**NA**L*VQ**K*****E**R**C**P**N**S	398	ArabSUP45
GSQFVKGFGGIGGILRYRVDFPQGEVYQGGDDEFFDLDDY	437	HuTB3/C11
*****	437	X1C11
*A*****AM*****K*N**EQLVDESE*-**Y**E**EGSDYDFI	437	YSUP45
*****CR*****L*****QL**MRTFD-ELS*G*VYE--DSD	435	ArabSUP45

FIG. 2 Alignment of the predicted amino-acid sequences of the human C11 protein (HuC11)⁷, human TB3-1 protein (HuTB3) deduced from the resequenced (this work) TB3-1 cDNA clone⁶, *X. laevis* C11 protein (X1C11)⁷, yeast sup45 (sup1) protein (YSUP45)¹⁰ and *A. thaliana* sup45-like protein (ArabSUP45; GenBank accession number X69375). Asterisks denote residues identical to the human TB3-1/C11 protein. Four peptides sequenced from rabbit 50K polypeptide (rab.eRF) are shown. Peptides a and b are identical or very similar to a fragment of human TB3-1/C11, *X. laevis* C11, *S. cerevisiae* sup45 and the *A. thaliana* sup45-like proteins. Peptide c is very similar to a fragment of human TB3-1/C11 and *X. laevis* C11 proteins, but differs considerably from yeast sup45 and *A. thaliana* sup45-like protein fragments. Peptide d is similar only to a fragment of the human TB3-1/C11 and *X. laevis* C11 proteins. Peptides a, b, and c plus d derived from rabbit eRF are scattered along the polypeptide chains of this protein family, indicating that the homology between rabbit eRF and the other proteins encompasses the entire length rather than a limited part of the polypeptide.

FIG. 3 SDS-PAGE (10% acrylamide) and immunoblot analysis of the *X. laevis* C11 (expressed in *E. coli*) and human TB3-1 (expressed in yeast) proteins. a, Coomassie blue staining; b, western immunoblot. M_r , protein markers; lane 1, *E. coli* cell lysate with the expressed C11; lane 2, the purified C11 protein (BSA was added for stabilization; upper band); lane 3, noninduced yeast cell lysate carrying the recombinant plasmid pTB3-1L1; lane 4, same as lane 3 but induced; lane 5, purified TB3-1.

METHODS. The blotted antigens were detected by anti-C11 antibodies using ECL (Amersham) according to the manufacturer's instructions. *X. laevis* C11 cDNA⁷ was inserted into the *Pst*I site of the pQE30 (QIAGEN) plasmid (pQE30-C11). The first eight amino acids at the amino terminus of the wild-type C11 were substituted by MRGSHHHHHGSASELGT-PGRPA in the His-tagged C11. *E. coli*, strain M15, was transformed with pQE30-C11 and induced to express the His-tagged C11. After elution of the protein from Ni-NTA resin with 0.25 M imidazole, DTT and BSA were added up to 2 mM and 0.2 mg ml⁻¹, respectively. The fractions containing C11 protein were combined, dialysed against buffer containing 50 mM Tris-HCl pH 7.5, 0.1 M KCl and 2 mM DTT, concentrated on Centricon 10 (Amicon) and stored at -80 °C. The *Eco*RI-*Eco*RI 2.3-kilobase (kb) fragment of the human TB3-1 cDNA⁶ was recloned into



3b) and with rabbit eRF (now shown) in immunoblots. Moreover, genetic complementation experiments point to the functional interchangeability of the *X. laevis* C11 and yeast sup45 proteins⁷.

The high conservation of the amino-acid sequence in this family is remarkable and may imply that the function of these proteins is critically important for cells. In this family, two proteins, rabbit eRF and yeast sup45, are involved in translation: biochemically purified rabbit eRF recognizes stop codons in ribosomes and catalyses peptidyl-tRNA hydrolysis¹⁵, whereas the yeast omnipotent suppressor sup45 is known to be involved in translation⁸⁻¹², and its association with translation termination has been discussed^{8,21}. The low abundance of sup45 protein²¹ in ribosomes is in line with the putative function of this protein as, in contrast to elongation factors, RF participates only once in the synthesis of a given polypeptide.

Although no function has so far been attributed to the human and *X. laevis* proteins, their structural similarity to rabbit eRF suggests that they are also eRFs. To confirm this, a *X. laevis* His-tagged C11 fusion protein was expressed in *E. coli*, affinity-purified (Fig. 3a), and tested in the *in vitro* RF assay; it possessed RF activity which was stop-codon-dependent, because the presence of non-stop (sense) codon abolished any RF activity (Table 1). Furthermore, the RF activity of the C11 was inhibited by anti-C11 antibodies; this inhibition is specific, as addition of

pTZ19 plasmid, yielding pTZ19-TB3-1, and the *Eco*RI-*Sall* (1.7 kb) fragment of the TB3-1 cDNA was inserted into the pSGL36 vector constructed on the basis of pJDB219 (ref. 25) and containing the *LEU2* gene and *ori* 2 μ yeast DNA. In this pTB3-1L1 construct, the TB3-1 cDNA was transcribed under the promoter and terminator sequences of the yeast acidic phosphatase gene, *PHO5*. *S. cerevisiae*, strain AH22 (MATa leu2-3, 112 his3 can1-II), was transformed with pTB3-1L1. Human TB3-1 expressed in yeast was purified by column chromatography as described for rabbit eRF (see Fig. 1 legend) except for omission of the Mono-Q column step.

anti-Eg5 antibodies did not significantly inhibit the RF activity. Two other affinity-purified His-tagged fusion proteins showed no RF activity in the *in vitro* RF assay (Table 1), indicating that the RF activity of the His-tagged C11 protein is not due to the presence of the His tail.

The full-length human TB3-1 cDNA clone⁶ was expressed in yeast and the TB3-1 protein was purified. SDS-PAGE yielded a dominant band of apparent M_r 50K (Fig. 3a) which coincided with the position of the *X. laevis* C11 protein. Purified TB3-1 protein tested in the *in vitro* RF assay possessed RF activity (Table 1).

Bacterial RF1, RF2 and mitochondrial RF1 are structurally related²², whereas RF3 (refs 2, 3) shows no similarity to these proteins. We failed to detect any obvious structural resemblance between the bacterial and mitochondrial RFs and eRFs. This may indicate that the mechanism of eukaryotic termination evolved independently of the prokaryotic counterpart, from another ancestor polypeptide. Alternatively, despite structural diversity at the level of the amino-acid sequence, the prokaryotic and eukaryotic RFs may be similar at the three-dimensional level. In prokaryotes, three RFs participate in translation termination whereas in mammals only one eRF has so far been detected^{5,13,14}. However, other, as yet unidentified, RFs may exist in eukaryotes. In view of the present results, we propose to designate this protein family (Fig. 2) as eRF1. □

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Structure of the human ADP-ribosylation factor 1 complexed with GDP

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ADP-ribosylation factors (ARFs) are essential and ubiquitous in eukaryotes, being involved in vesicular transport and functioning as an activator of phospholipase D (refs 1, 2) and cholera toxin^{3,4}. The functions of ARF proteins in membrane traffic and organelle integrity^{5,6} are intimately tied to its reversible association with membranes and specific interactions with membrane phospholipids. One common feature of these functions is their regulation by the binding and hydrolysis of GTP. Here we report the three-dimensional structure of full-length human ARF1 (*M*_r 21,000) in its GDP-bound non-myristoylated form. The presence of a unique amino-terminal α -helix and loop, together with differences in Mg^{2+} ligation and the existence of a non-crystallographic dimer, set this structure apart from other GTP-binding proteins. These features provide a structural basis for the GTP-dependent modula-

tion of membrane affinity, the lack of intrinsic GTPase activity, and the nature of effector binding surfaces.

The structure of ARF1 has been determined to a resolution of 2.0 Å by X-ray diffraction using multiple isomorphous replacement (Table 1). The folding motif of ARF1 is that of an α/β protein composed of a mixed β -sheet core surrounded by α -helices (Fig. 1). This core is reminiscent of Ras p21 (ref. 7), with which it shares a 21% sequence identity⁸, the nucleotide-binding domain of the α -subunit of the G protein transducin ($G_{i\alpha}$)⁹, and domain 1 of the elongation factor EF-Tu (ref. 10). A number of novel structural features are found in ARF1 that distinguish this protein from other GTP-binding proteins. The most important is the presence of an N-terminal α -helix (αA) and connecting loop (L1). The sequence of this helix and loop segment has no counterpart in the sequences of *ras* family members¹¹, but does appear in the sequences of G-protein α -subunits⁹. This N-terminal segment is critical to ARF1 functions both *in vivo* and *in vitro*¹². The location of the N-terminal helix and loop gives the first structural indication of the biochemical mode of action of ARF1 in relation to three essential components of its function, namely membrane affinity, nucleotide-induced conformational change, and effector interactions. The N-terminal αA (Fig. 2a) is amphipathic, lies in a groove parallel to the C-terminal αF , and is held in place largely by hydrophobic forces. Helix αA acts as an anchor that localizes the biologically important myristoyl group when it is covalently bound to the N-terminal glycine¹³. In contrast to the residues of αA , the residues of L1 connecting this helix to $\beta 1$ are rather disordered in the structure, indicating considerable flexibility. A number of residues in L1, together with a lysine from the neighbouring β -turn L5 and an arginine from αF , contribute to a cluster forming

FIG. 1 Stereo ribbon diagram of the ARF1 crystallographic dimer. The two ARF1 molecules are related to each other by a non-crystallographic twofold axis in the asymmetric unit of the unit cell. For each ARF1, helices αA , αB and αF lie below the plane of the β -sheet. αC and αD lie above the plane. $\beta 2$ and αE lie in the plane of the β -sheet forming its outer edges. The bound GDP is depicted as a ball-and-stick model, and the magnesium ion is shown in yellow. The image was rendered using RAYSCRIPT²⁶ (this program uses the output of MOLSCRIPT²⁷ as input for RAYSHADE²⁸). $\beta 2$ represents an additional secondary structural feature not seen in Ras. $\beta 2$ also forms the dimer interface region through main chain hydrogen bonds between the $\beta 2$ strands. Residues of $\beta 2$ and L4 correspond to the effector loop in Ras by sequence comparison. In contrast to the extended effector loop (L2) that is in proximity (10.5 Å between Thr 35 O γ and P of β -phosphate) to the nucleotide in Ras, $\beta 2$ of ARF1 leads away, placing Thr 48 in L4 much further (20.5 Å between Thr 48 O γ and P of β -phosphate) from its nucleotide. Although the GDP-Mg²⁺ complex is extensively exposed to solvent in the monomer, it is buried in the dimer. The extent of the protein interface is much larger than the hydrogen bonding of the β -sheet formed by $\beta 2$ and $\beta 2'$. The additional interfacial residues are partially buried by contact with the other monomer. Using QUANTA²⁹, a calculation of the solvent-accessible surface area was made for the dimer and the monomers individually. From these calculations, an estimated area of 700 Å² of the 8,700 Å² available solvent-accessible surface of each monomer is buried by the other monomer in the dimer. Thus, the dimer is held together not only by the continuation of the β -sheet, but also by van der Waals interactions and hydrophobic forces.

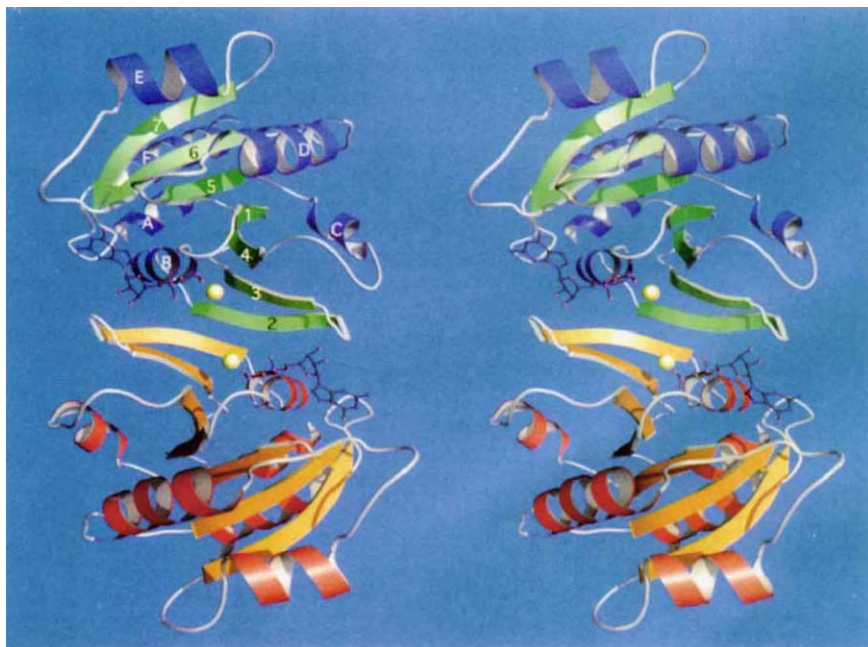
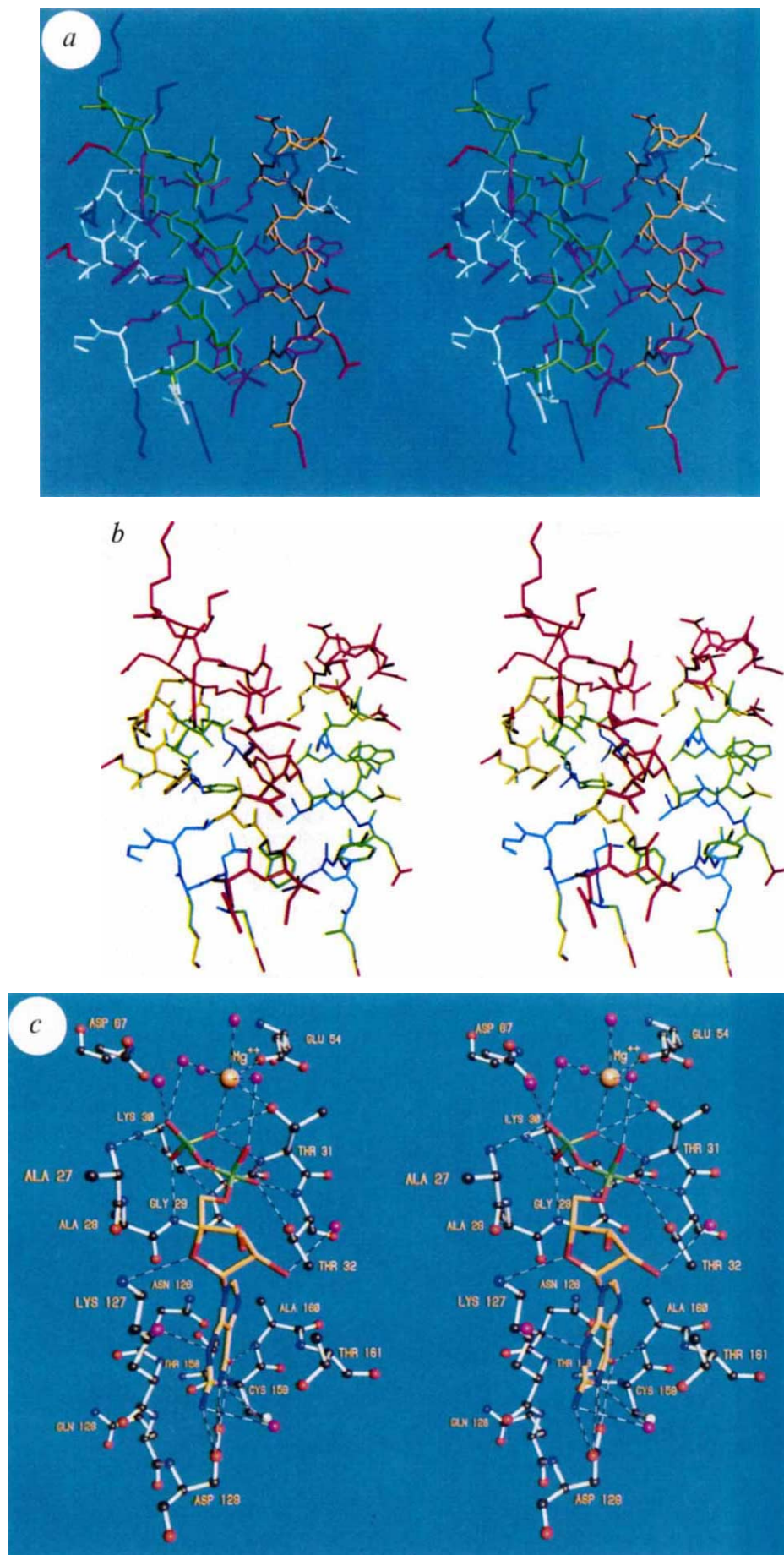
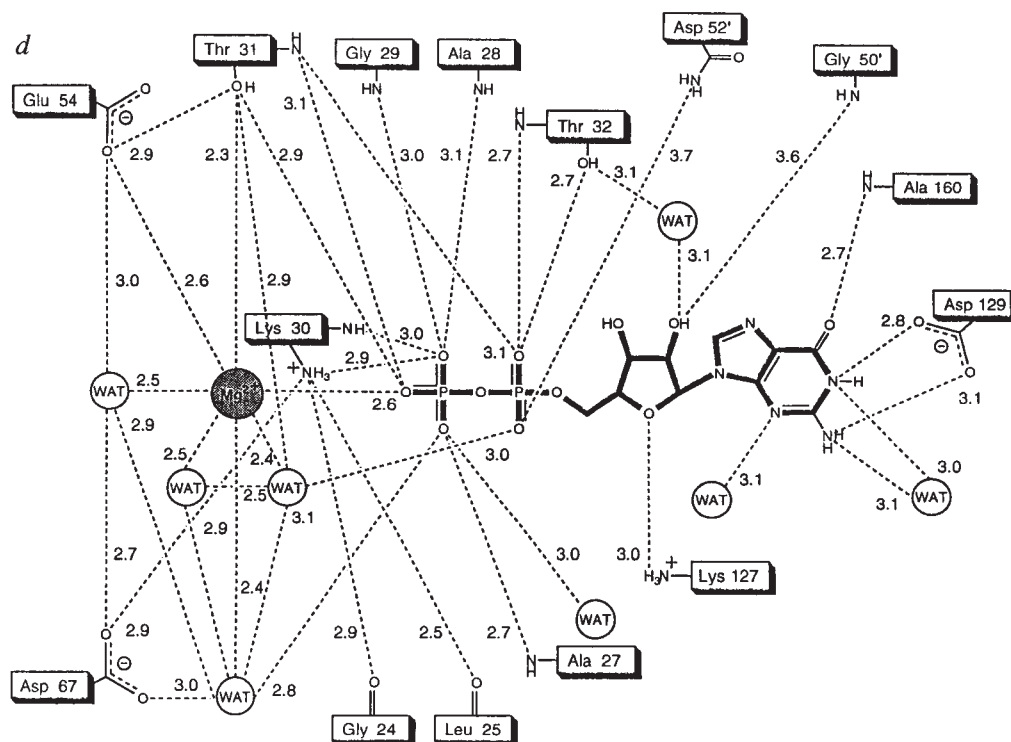


FIG. 2 *a*, Stereo representation of the position of α A and L1 (residues 2–16), the positive patch and residues lining the cleft that holds the N-terminal helix α A. The backbone atoms of α A, L1 and part of β 1 are shown in green. The backbone of the C-terminal helix α F is in yellow, and the backbones of L3 and L5 are in white. Hydrophobic residues interacting in the cleft that holds α A are in magenta. Positively charged residues are in blue. Negatively charged residues are in red. Note the formation of a positive patch by residues from L1 (K10, K15, K16), L5 (K59), and α F (R178). This includes the C-terminal Lys which could easily rotate around its ϕ -bond to bring the N_ϵ close to the area of the positive patch. *b*, Stereo representation of the same region as in *a*, but coloured by temperature factor. Blue represent segments of lower ($<25 \text{ \AA}^2$) flexibility, and red represent regions of highest ($>60 \text{ \AA}^2$) flexibility. *c*, Stereo model with hydrogen bonding indicated, and *d*, (overleaf) schematic diagram with distances (in $\text{\AA}$, where $1 \text{ \AA} = 0.1 \text{ nm}$) of the nucleotide and Mg^{2+} bound to ARF1. The GDP interacts with some of the residues predicted from the association of a GDP with RAS. However, some interactions are very different. The guanine base interacts with the TCAT box (158-TCAT-161; L12) and the highly conserved guanine specificity region⁷ (126-NKXD; L10). Asn 126 hydrogen-bonds with part of the Walker sequence (Ala 28 C=O) and two residues of the TCAT box (Thr 158 O γ and Ala 160 NH). The α - and β -phosphates of GDP lie across L2 and the N terminus of α B (24-GLDAAGK-30; the Walker sequence). Asp 26 (homologous to Gly 12 in Ras) makes a salt bridge to residue Arg 99. Both of these residues are absolutely conserved in all ARFs (R. A. Kahn, unpublished). In contrast to the guanine base, the ribose ring makes only one hydrogen bond to its own monomer (Lys 127 N ζ). Interestingly, the ribose 2' oxygen is 3.6 $\text{\AA}$ from the amide of Gly 50' in L4' of the other monomer. One α -phosphate oxygen is hydrogen-bonded between the Thr 31 NH, and the amide and O γ of Thr 32. The other α -phosphate oxygen, however, is within 3.7 $\text{\AA}$ from the Asn 52' N δ in β 3', also of the other monomer. In contrast to the α -phosphate, the β -phosphate is held in place by an intricate network of hydrogen bonds and metal ligation. Also notable is the preponderance of partial negative charges in the phosphate-binding region, which may explain the higher affinity of GDP over GTP¹⁸. Drawings in *a*, *b* and *c* were rendered using RAYSCRIPT²⁶; *d* was drawn with CSC ChemDraw³⁰.





a positively charged patch on the surface (Fig. 2a). This patch may be important for ARF1 association with the membrane.

Binding of GTP mediates membrane localization¹² and is accompanied by a conformational change¹⁴. If association with a membrane is accomplished by insertion of the myristoyl group into the bilayer, then this conformational change must involve movement of αA and L1. The flexibility of L1 (Fig. 2b) combined with the repeating helical pattern (LFXX) in the sequences¹⁵ of αA and L1, and their location relative to the overall structure, led us to speculate that the residues of L1 will form a continuation of αA in response to nucleotide exchange. This would make the bound myristoyl moiety available for membrane insertion. The process could be promoted by the interaction of the positively charged patch with a negatively charged surface of the membrane bilayer. This is analogous to the conformational change that occurs in influenza haemagglutinin in response to the change in pH that induces membrane fusion¹⁶. In addition, biochemical studies indicate that nucleotide exchange is facilitated in an ARF1 mutant lacking the first 17 amino acids¹². Therefore it will be possible to speculate on the interaction between an exchange factor and the N terminus of ARF1-GDP, based on this structure.

The magnesium ligation has several unexpected features (Fig. 2c, d and Fig. 3). The differences in ligation of the magnesium ion in ARF1, compared to Ras, are linked to a shift in registration of $\beta 4$ by two amino acids towards the C terminus relative to its counterpart ($\beta 3$) in Ras. This unexpected shift particularly affects the position of Gln 71. The homologous residue in Ras (Gln 61) appears to be critical for the hydrolysis of GTP in Ras¹⁷. The Gln 71→Leu mutant of ARF1 is lethal to mammalian cells and yeast and is defective in GTP hydrolysis stimulated by the ARF GTPase-activating protein (GAP)^{5,6}. The shift in registration causes a displacement of Gln 71 such that its alpha carbon is ~ 5 Å further away from the putative binding site of the γ -phosphate than Gln 61 is in Ras (Fig. 3b). This configura-

tion of the active site, unique to ARF1, may be the explanation for the lack of intrinsic GTPase activity of the isolated protein^{14,18}. Further speculation on the consequences of the β -strand shift awaits the ARF1-GTP structure.

ARF1 appears as a dimer in the asymmetric unit of these crystals (Fig. 1). The two monomers interact through two backbone hydrogen bonds, forming a continuous β -sheet through the antiparallel alignment of $\beta 2$ of each molecule. $\beta 2$ constitutes an additional, new β -strand not found in Ras or the G_α subunits. One of the consequences of this dimerization is the close proximity of the evolutionarily conserved sequence of loop L4 (47-PTIG-50) from one ARF1 monomer to the nucleotide of the other monomer (Figs 1 and 2c, d). The residues of ARF1 $\beta 2$ and L4 are equivalent to the extended effector loop in Ras (L2) which is known to interact with RasGAP and neurofibromin¹⁹. A comparable function can be envisaged for this region in ARF1, and we speculate that this type of dimer interface represents a model for interactions between ARF1 and other proteins. The fact that the most substantial structural change between ARF1 and Ras is seen in the area of ARF1 corresponding to the known effector site of Ras, suggests that this area is also responsible for an effector interaction in ARF1. That effector interaction would appear to be modulated by differences not only in sequence but also in structure.

ARF proteins make a large number of specific, high-affinity interactions with ligands such as GDP, Mg^{2+} and phospholipids, and with other proteins including G_s , cholera toxin, PLD and ARF-GAP. Directed mutagenesis and structural analysis have begun to probe these interactions in detail. The structure of the ARF1-GTP complex is important to the interpretation of these experiments. But differences between the GDP- and GTP-bound forms of Ras will not be useful in predicting the structure of ARF1-GTP. The observed differences between the ARF1 and Ras structures call into question the assumption that similarity of sequence can be used as a guide to the conservation of the

FIG. 3 *a*, Stereoview of the solvent-flattened MIR electron density map at a resolution of 2.5 Å contoured at the 1σ level around strand $\beta 4$. The map unambiguously shows the position of Trp 66 which establishes the amino acid registration for this strand. *b*, Position of Gln 71 and magnesium ligation in ARF1 relative to Gln 61 and magnesium ligation in Ras in reference to the bound GDP. The main-chain atoms of $\beta 3$, $\beta 4$ and $\beta 1$ of ARF1 (yellow) are superimposed on the main chain atoms of $\beta 2$, $\beta 3$ and $\beta 1$ of Ras (red) in the active-site region of these two proteins. The side chains of residues Glu 54 (orange), Asp 67 (blue) and Gln 71 (magenta) of ARF1 and the sequentially equivalent side chains of residues Asp 38 (orange), Asp 57 (blue) and Gln 61 (magenta) of Ras are shown. Only the diphosphate of GDP (green) is shown for clarity. The distance between the Gln 71 O ϵ and the β -phosphate P atom is ~ 17 Å, ~ 5 Å longer in ARF1 than the comparable distance for Gln 61 in Ras. A sequence alignment³¹ of $\beta 4$ (ARF1) and $\beta 3$ (Ras), predicts that Asp 67 of ARF1 should be the equivalent magnesium ligand to Asp 57 of Ras:

```

54ETVEYKN ISFTVWDVGG Q71 ARF1
... .. :...:|...|
44VVIDGET CLLDILD TAG Q61 Ras

```

Homology is based on evolutionary distance of amino acids³¹: single dots indicate distant homologues, two dots indicate recent homologues, and lines indicate identities. This alignment would place Gln 71 (ARF1) and Gln 61 (Ras) at an analogous position relative to the β -phosphates in the two proteins. Such a location for Gln 71 could lead to an intrinsic GTPase activity, as in Ras³², but there is no intrinsic GTPase activity detected for ARF1^{14,18}. But the structural alignment of these two strands is unexpectedly different (ARF1, upper; Ras, lower sequence):

```

       $\beta 3$             $\beta 4$ 
54ETVEY      KN ISFTVWDVGGQ71
36IEDSYRKQVVIDGETCLLDILD TAGQ61
       $\beta 2$             $\beta 3$ 

```

Asp 67 in ARF1 serves the same role as suggested for Asp 57 in Ras³³, which is to function as an indirect metal coordinator providing water as a ligand. However, Asp 67 does so originating from a different position than Asp 57, namely two residues further along $\beta 4$. Surprisingly, Glu 54 (ARF1) reaches from $\beta 3$ into the position predicted by sequence alignment for Asp 67 (ARF1). Glu 54 (ARF1) is clearly a direct ligand to the metal. The structural equivalent of Glu 54 (ARF1) is Asp 38 (Ras) which is oriented away from the metal and not at all associated with it in Ras. *c*, The changes of the relative alignments of the two strands associated with Gln 71 ($\beta 4$ in ARF1) and Gln 61 ($\beta 3$ in Ras) are accompanied by differences in the coordination sphere of the magnesium ion. The most prominent difference in ligands involves Glu 54 from $\beta 3$ which is a direct magnesium ligand in ARF1, rather than the expected Asp 67 from $\beta 4$ (indirect through water molecules), as based on sequence alignment. RAYSCRIPT²⁶ was used for depicting *a* and *b*; *c* was drawn with CSC ChemDraw³⁰.

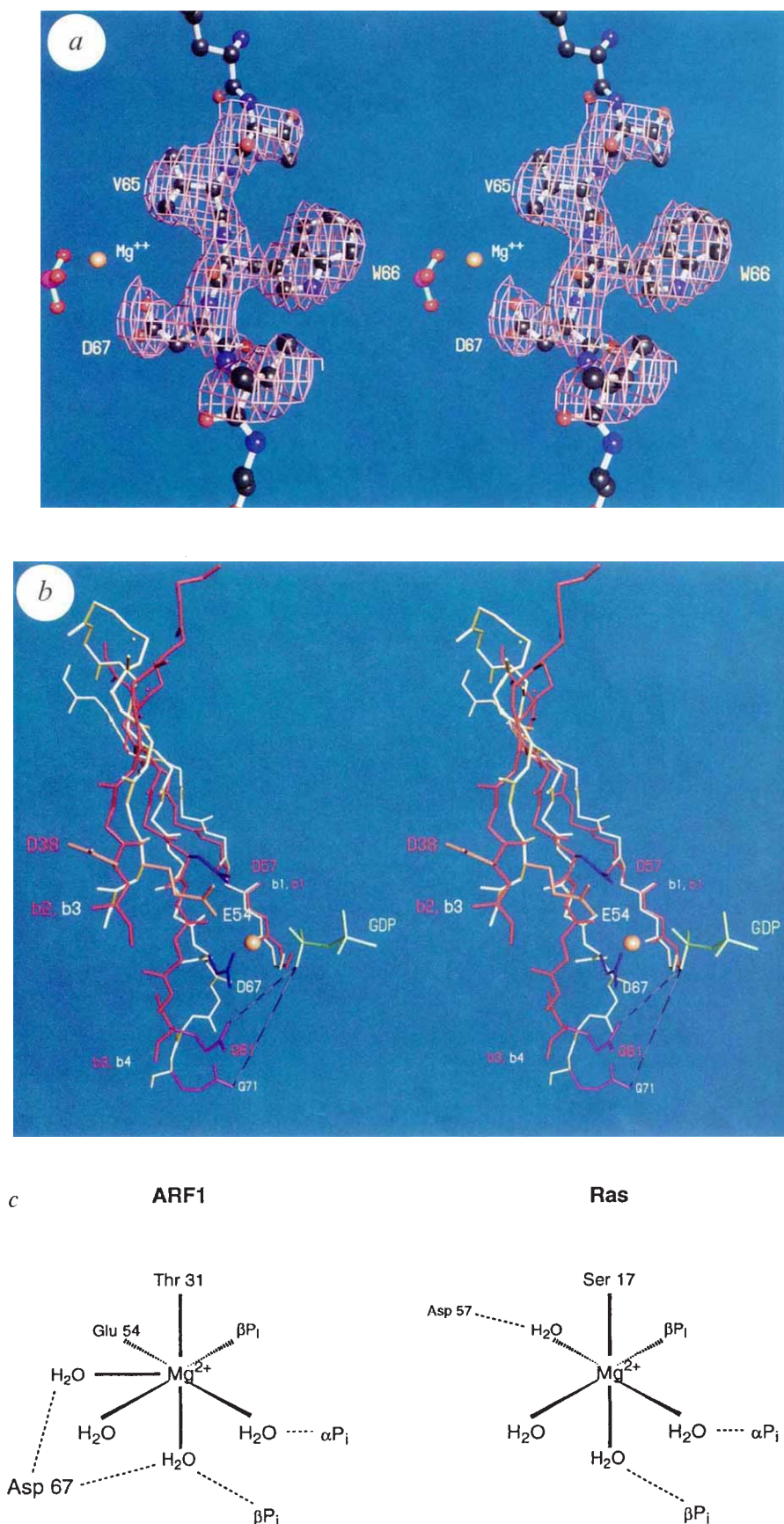


TABLE 1 Statistics for X-ray structure determination

Crystal parameters								
Type	Native		MAC		EMP			
Space group	C2		C2		C2			
Unit cell	$a=122.01$ $b=45.92$ $c=91.78$		$a=122.26$ $b=45.50$ $c=91.81$		$a=122.05$ $b=44.81$ $c=91.35$			
	$\gamma=133.51$		$\gamma=133.43$		$\gamma=133.25$			
Diffraction data								
Data set	No. crystals used	Resolution (Å)	Measured reflections	Unique reflections	Completeness (%)	R_{merge} (%)*	MFID†	
Native	1	2.0	46,853	19,925	82	7.79	—	
MAC	1	2.0	48,970	20,166	84	5.12	0.21	
EMP	1	2.0	48,026	18,132	76	5.85	0.23	
MIR Phasing								
Derivatives	No. of sites	Resolution (Å)	R_K (%)‡	Phasing power§				
MAC	4	2.8	11.8	2.32				
EMP	4	2.8	14.7	2.04				
Structure refinement								
	R_{overall}	R_{free}	Resolution (Å)	Reflections	Protein atoms	GDP atoms	Mg^{2+} atoms	Water atoms
All data	21.3	30.9	2.0	19,731	2,839	56	2	95
Data $I > 2\sigma(I)$	19.5	29.2	2.0	15,409	2,839	56	2	95
R.m.s. bond deviation from ideal: 0.018 Å								
R.m.s. angle deviation from ideal: 1.7°								

Recombinant human ARF1 was expressed and purified essentially according to ref. 18. ARF1 was crystallized by the hanging drop method at room temperature against a solution containing 13% polyethylene glycol (PEG) 8000, 150 mM CaCl_2 and 100 mM cacodylate (pH 6.5). Heavy-atom derivatives were obtained by soaking crystals in 1–2 mM mercury acetate (MAC) or ethyl mercury phosphate (EMP) solutions containing 20% PEG 8000, 75 mM CaCl_2 and 100 mM cacodylate (pH 6.5) for 12–18 h in the dark. Diffraction data were collected on a Rigaku R-Axis II image plate detector using 0.3-mm collimated, monochromized Cu K α radiation from a Rigaku RU-200 rotating anode generator running at a power of 50 kV and 150 mA. The heavy-atom positions were refined to a resolution of 2.8 Å with the TENEX²⁰ program, giving an initial figure of merit of 0.66. Derived phases were improved and extended with solvent flattening to a resolution of 2.5 Å using all data, with the programs BCWANG²¹ and SQUASH²², without non-crystallographic averaging. The solvent-flattened map showed improved connectivity, but was substantially the same as the initial MIR map. These solvent-flattened phases were used to generate an initial protein electron density map used for modelling. This map was sufficiently clear to identify unambiguously and build the secondary structural elements and to position side chains of bulky amino acids. Figure 3a is an example of the quality of that initial electron density map. A partial three-dimensional model was fitted to the electron density using the program O²³. Additional improvement in the map was obtained by combining initial MIR phases and the partial model with the program COMBINE²⁴. The two monomers were built and refined independently of each other using alternating rounds of simulated annealing (X-PLOR²⁵) and manual rebuilding. A group B-factor was applied in the initial stage of refinement. Individual B-factors were applied after the R-factor of the model dropped below 25%, using all data to 2 Å resolution. The monomers in the dimer are very similar in structure, with the r.m.s. deviation for the position of α -carbons, excluding loops L1 and L6 (residues 10–16 and 72–74), of 0.49 Å. The electron density for both loops is poorly defined and allows residues to be modelled only with a high-temperature factor.

* $R_{\text{merge}} = \sum |F^2(i)/G(i) - \langle F^2(h) \rangle| / \sum F^2(i)/G(i)$, where $F^2(i)$ is the i th integrated intensity, $\langle F^2(h) \rangle$ is the averaged integrated intensity with Miller indices $h = (hkl)$, and $G(i)$ is the inverse scale factor.

† MFID (mean fractional isomorphous difference) = $\sum (|F_P| - |F_{PH}|) / \sum (|F_P|)$, where F_P and F_{PH} are the structure factor amplitudes for the native and heavy-atom derivative, respectively.

‡ $R_K (R_{\text{Kraut}}) = \sum |F_{PH} - F_{PH(\text{calc})}| / \sum |F_{PH}|$, where F_{PH} is the structure factor amplitude for the heavy-atom derivative and $F_{PH(\text{calc})}$ is the calculated heavy-atom structure factor amplitude.

§ Phasing power = $\langle F_H \rangle / \langle E \rangle$, where $\langle F_H \rangle$ is the r.m.s. of the heavy-atom structure factor amplitude and $\langle E \rangle$ is the r.m.s. lack of closure error.

detailed structure of other GTP-binding protein active sites and effector regions. □

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Self-assembling organic nanotubes based on a cyclic peptide architecture

M. Reza Ghadiri, Juan R. Granja, Ronald A. Milligan, Duncan E. McRee & Nina Khazanovich

Nature 366, 324–327 (1993)

IN this Letter important references to earlier works were inadvertently left out. De Santis *et al.*, in the context of a theoretical conformational analysis of linear regular L,D polypeptides, suggested formation of parallel and antiparallel cylindrical structures through interannular association of β -type rings¹. Synthesis of some cyclic oligopeptides with S_{2n} symmetry, with the construction of tubular structures as the stated goal, was first reported by Tomasic and Lorenzi², when the possibility of tubular aggregate formation was tentatively assigned based on the solubility profiles and infrared analysis². We thank G. P. Lorenzi for kindly bringing these references to our attention. □

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A deep earthquake aftershock sequence and implications for the rupture mechanism of deep earthquakes

Douglas A. Wiens, Jeffrey J. McGuire, Patrick J. Shore, Michael G. Bevis, Kitlone Draunidalo, Gajendra Prasad & Salmone P. Helu

Nature 372, 540–543 (1994).

THE numbers on the depth scale in Fig. 3a and b in this Letter were incorrect, making the total width of the aftershock zone look as though it was 25 km, rather than 50 km as correctly reported in the text. The numbers on the y-axis of Fig. 3a should

read 555, 575 and 595 km (not 565, 575 and 585 km as published); on the y-axis of Fig. 3b they should be 545, 565, 585 and 605 km (instead of 560, 570, 580 and 590 km). □

A TCP1-related molecular chaperone from plants refolds phytochrome to its photoreversible form

Eckart Mummert, Rudolf Grimm, Volker Speth, Christoph Eckerskorn, Emile Schiltz, Anthony A. Gatenby & Eberhard Schäfer

Nature 363, 644–648 (1993)

THE following correction concerns only the contribution to this paper of E.M., R.G. and E. Schäfer. We purified a protein of M_r 60,000 (60K) from oat seedlings that possesses an oligomeric structure of 600K and is partially similar to the cytosolic TCP1 proteins from certain eukaryotes. Specific oligonucleotides were generated following internal peptide sequencing and were used to screen a cDNA library from oats. Ten clones were characterized by sequencing, all of them containing the TCP1-related internal sequenced published before our paper. The clones belong to two subclasses which mainly differ in the deduced amino-terminal sequence of the mature proteins: ALESA for one and ELESF for the other class.

All clones are very similar to β -glucosidase from *Zea mays* and glucosidases and galactosidases from other organisms¹, but show no overall homology to TCP1 genes. The purified 60K protein has β -glucosidase and β -galactosidase activity, which was recently confirmed by Rüdiger and co-workers².

To test the putative chaperone activity of the 60K protein, we purified it in high amounts using a purification procedure slightly different from that published in our paper. In contrast to the data published in our paper, this purified fraction is not able to refold phytochrome to its photoreversible form. On the other hand, it can prevent the aggregation of denatured citrate synthase in a light-scattering assay. Because the purified fraction contains traces of an 800K protein strongly crossreacting with antibodies raised against chloroplast cpn60 or against *E. coli* GroEL, we cannot definitely exclude that the apparent chaperone activity is caused by the contaminating proteins and not by the 60K β -glucosidase. □

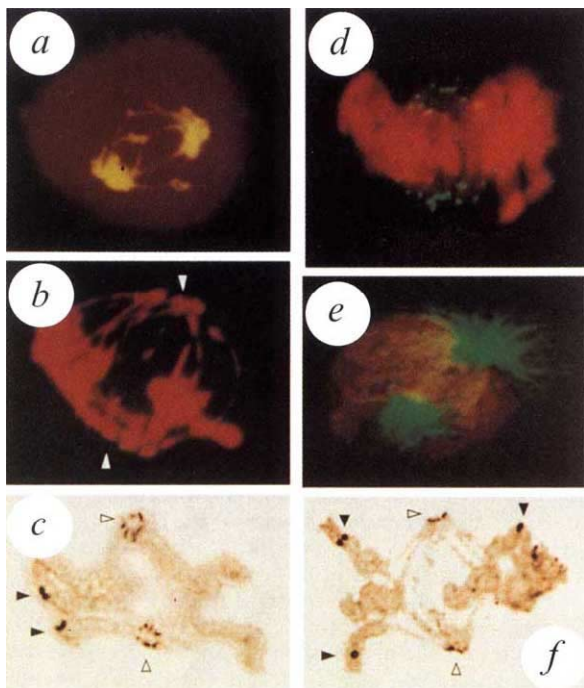
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A topoisomerase II-dependent G2 cycle checkpoint in mammalian cells

C. Stephen Downes, Duncan J. Clarke, Ann M. Mullinger, Juan F. Giménez-Abián, Andrew M. Creighton & Robert T. Johnson

Nature 372, 467–470 (1994)

DURING production a stray line was introduced across panel *c* of Fig. 4 of this Letter. The figure is reproduced below. □



Alternative pathway of insulin signalling in mice with targeted disruption of the *IRS-1* gene

Eiichi Araki, Myra A. Lipes, Mary-Elizabeth Patti, Jens Claus Brüning, Burritt Haag III, Randall S. Johnson & C. Ronald Kahn

Nature 372, 186–190 (1994)

THE contents list in this (the 10 November) issue included a typographical error. □

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